Inhibins in Normal and Down's Syndrome Pregnancies

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To my wife Karen, and our two wonderful children, Duncan and Ailsa.

I hereby declare that this thesis has been composed by myself and that the work described in the thesis was undertaken by myself, through collaboration with other parties as acknowledged in the text. Further, I confirm that the thesis has not been submitted for any other degree, diploma or professional qualification.

Euan M Wallace
April 1997

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Abstract of Thesis

Down's syndrome (trisomy 21) is the single most common recognised cause of mental handicap in the United Kingdom. Not surprisingly therefore, a number of approaches have been developed to screen and diagnose Down's syndrome pregnancies prenatally, affording individuals the opportunity to terminate the pregnancy if desired. The most effective and widely used of these approaches currently involves amniocentesis targeted by second trimester screening based upon maternal age in combination with the measurement of various factors in maternal serum. Such an approach will detect approximately 60-70% of Down's affected pregnancies with an overall amniocentesis rate of 5%. However, this of course means that a third of affected pregnancies remain undetected and that many women expose their pregnancy to the risks of amniocentesis-related miscarriage. Screening with improved sensitivity and specificity is therefore desirable.

This thesis describes a series of clinical and laboratory studies examining the value of inhibins as novel prenatal markers of Down's syndrome. Inhibins are a family of glycoprotein proteins initially identified in gonadal tissue but known to be secreted by the placenta in pregnancy. The ontogeny of inhibin secretion in early pregnancy is described in detail comparing various pregnancy compartments in both normal and Down's syndrome pregnancies. It is shown that maternal serum levels of inhibin-A, one of two possible fully processed inhibin dimers, are significantly elevated in Down's syndrome in both the first and second trimesters of pregnancy. Furthermore, mathematical modelling studies with other established prenatal markers (maternal age, human chorionic gonadotrophin and alpha fetoprotein) suggests that inhibin-A could increase second trimester detection rates by as much as 20%.

Studies on amniotic fluid revealed that, unlike maternal serum in which only inhibin-A is detectable, both dimers, inhibin-A and inhibin-B, were present raising the possibility that the fetal membranes secrete significant amounts of inhibin. The lack of a relationship between inhibin-A levels in paired serum and amniotic fluid samples supported this hypothesis, representing a significant re-appraisal of the previous understanding of inhibin biology in pregnancy.

Studies of amniotic fluid inhibin levels in Down's syndrome showed that inhibin-A levels are significantly lower than normal, the opposite of serum. This suggests that the fetal membranes not only secrete different inhibins from the placenta but that the secretion is differentially controlled.

These studies have therefore described, in detail, the biology of specific inhibin forms in normal and aneuploid pregnancy demonstrating inhibin-A to be the second most valuable serum marker of Down's syndrome. Novel insights into normal placental endocrinology have also been afforded by the studies with the promise of new initiatives in Down's syndrome screening and conditions with related endocrinology.

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A number of individuals and institutions have had significant roles in the studies detailed in this thesis. Without these people the work undertaken would not have been possible and I am indebted to them for their time, skills and willingness to collaborate or assist with my own personal research interests. In recognition of the assistance that I have had I am very grateful for the opportunity to formally acknowledge these individuals here, as well as the specific references in the relevant sections of the text of the thesis.

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Ethical Considerations and Procedures

In line with the Helsinki Declaration, some of the studies detailed in this thesis necessitated approval by an independent human research and ethics committee. For the studies using samples generated in Edinburgh, where required and as detailed in the thesis, application for consideration and approval was made to the Reproductive Medicine subcommittee of the Lothian Health Research Ethics Committee. Formal, written approval was obtained in each case prior to the commencement of the work. Details of each approval, if required, are available from the above committee.

For other collaborative studies, using samples generated elsewhere, ethical approval had been obtained, if required, by the collaborating scientist. Details of these approvals are available only from these individuals or from the relevant ethical committee.

Chapter One

Inhibins and Aneuploidy: an Introduction

1.1 Objectives

The studies detailed in this thesis were undertaken to explore the biology of inhibins in normal and abnormal pregnancy. As will become apparent, until very recently, the understanding of inhibin biology in man was based largely upon assay technology that was unable to differentiate between the various inhibin forms that are known to exist in biological fluids. Thus, as discussed in this and subsequent chapters, data from *in vitro* and animal studies were not always consistent with *in vivo*, clinical human data. The advent of novel sensitive and specific assays however, has afforded fresh clarity to the areas of controversy and allowed new insights into the understanding of inhibin biology.

The focus of this thesis has been to describe the ontogeny of the inhibins in early normal pregnancy thereby allowing a comparison with abnormal pregnancy, in particular with pregnancies complicated by Down's syndrome (trisomy 21). The chapters have been ordered to clarify such comparisons although, as will be apparent, the studies underlying the chapters were not necessarily performed in the order they are presented.

It is hoped that the data contained in this thesis, and resultant peer-reviewed publications, have not only afforded some clarification of the basic biology of inhibins in pregnancy but have also afforded novel observations that are of direct and immediate relevance to modern clinical obstetric practice. The following sections in this chapter will act as way of an introduction to inhibins in general, inhibins in pregnancy and maternal serum screening for Down's syndrome, as preparation for the studies and discussions that follow in subsequent chapters.

1.2 Inhibins and related proteins: a brief history

It is now over seventy-five years since it was first observed that impaired testicular function was associated with changes in the anterior pituitary gland (Mottram and Cramer 1923) and that those changes probably related to release of the pituitary from the suppressive influences of a non-steroidal regulator (McCullagh 1932). Indeed, it was McCullagh who coined the name "inhibin" for the as yet unidentified hormone. Subsequent to these early *in vivo* observations there was little further progress until the 1970s when numerous studies showing that follicle-stimulating hormone (FSH) secretion from the pituitary could be selectively suppressed by various testicular fluids

and extracts were reported (Franchimont *et al* 1972, Setchell *et al* 1974, Keogh *et al* 1976). However, more rapid progress became possible when it was recognised that, in addition to the testis and testicular extracts, follicular fluid contained large amounts of inhibin-like activity (de Jong and Sharpe 1972), because, compared to testes, bovine and porcine follicular fluid was readily available in very large volumes (Burger 1992).

In 1985 the successful isolation and partial characterisation of inhibin was reported simultaneously and independently by different groups (Robertson *et al* 1985, Ling *et al* 1985). During the numerous extraction and purification steps developed to isolate inhibin, it became clear that another protein, similar to inhibin, existed but that this protein stimulated FSH secretion rather than suppress it (Vale *et al* 1986). Maintaining McCullagh's terminology this protein was called "activin". Despite the extremely slow progress made between the 1930s and the 1980s, within three years of the first isolation of inhibin, the complete family of proteins had been described and named (Burger and Igarashi 1988), functions attributed (Ying 1988), immunoassays developed (McLachlan *et al* 1986, Bicsak *et al* 1986,Hasegawa *et al* 1988, McNeilly *et al* 1988), specific binding proteins identified (Ying 1988) and messenger ribonucleic acids (mRNAs) for the three subunits discovered in many and diverse tissues (Meunier *et al* 1988). By the close of the 1980s it was clear that the "inhibins" represented a significant new family of proteins with diverse functions throughout mammalian reproduction.

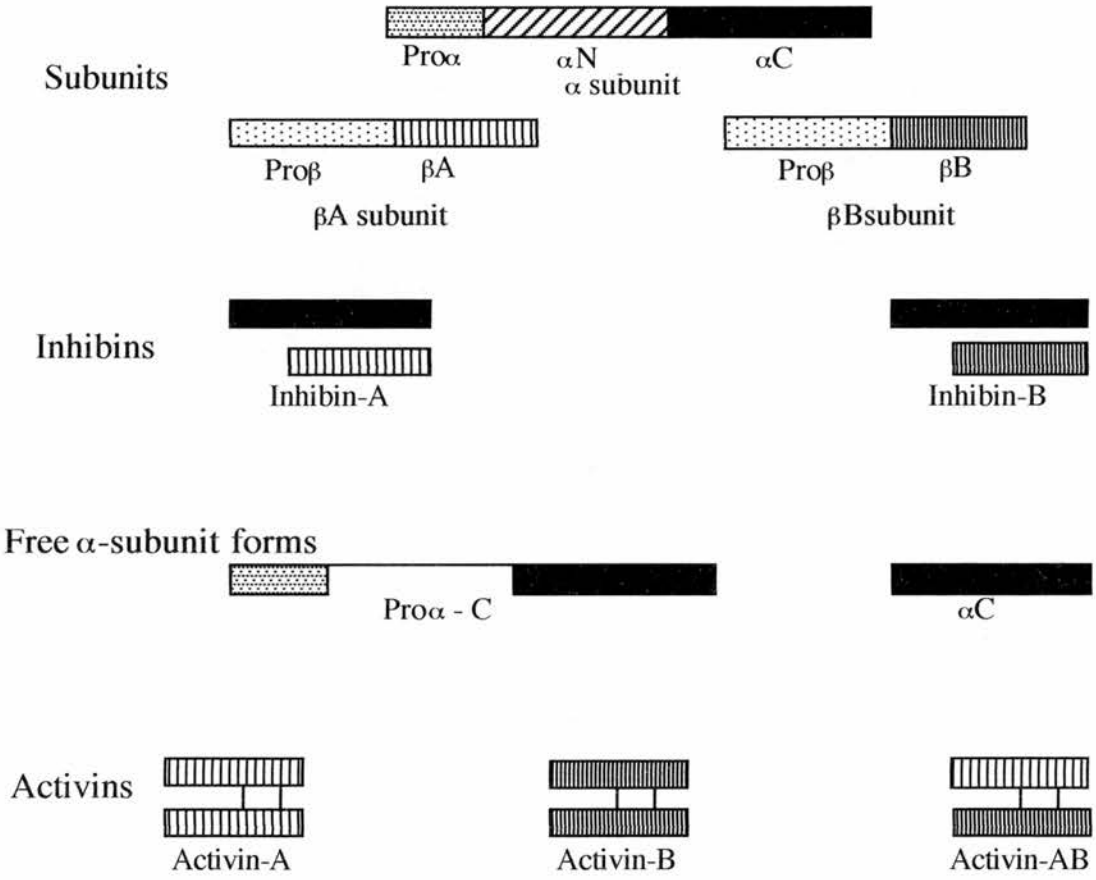
1.3 Inhibins: structure and detection

Inhibins and activins are members of the transforming growth factor-β (TGF-β) superfamily, a group of structurally similar but functionally diverse growth factors (Massague 1990, Kingsley 1994). Mature inhibin is a 31-32kDa heterodimeric glycoprotein composed of an α-subunit and one of two possible β-subunits, β_A or β_B, joined by disulphide bridges (Burger and Igarashi 1988). Thus, two 32kDa inhibins exist: inhibin-A (α-β_A) and inhibin-B (α-β_B). Activin is a homodimer of the β-subunits and therefore three possible species exist: activin-A (β_A-β_A), activin-B (β_B-β_B), and activin-AB (β_A-β_B) (Ying 1988). (Figure 1.1, page 5)

The initial purification of inhibin from bovine follicular fluid however, revealed a 58kDa protein and it was only subsequently that a smaller 31kDa species was isolated (Robertson *et al* 1985), the difference arising from cleavage of a 43kDa α-subunit precursor. A number of different molecular weight inhibin species was also reported in

bovine follicular fluid (Miyamoto *et al* 1986) and more recently Robertson and his colleagues have described in detail the numerous and various forms of inhibin in human serum (male and female) and human follicular fluid (Robertson *et al* 1995, Robertson *et al* 1996). These different molecular weight species relate to unprocessed and partially processed inhibin α-subunits, either free or dimerised to a β-subunit. The mature α-subunit is termed αC but this is processed from a translated pre-pro-α protein, giving rise to various α-subunit precursors: proαNαC, proαC and a cleavage product αN. β-subunits are also produced as pre-pro-proteins, processed to the mature β-subunit.

Figure 1.1
Subunits and forms of inhibins and activins.



It is currently understood that the inhibin subunit monomers (Ying 1988) and probably the higher molecular weight dimers (Mason *et al* 1996) are biologically inactive. Only two studies have reported the inhibin forms present in pregnancy sera. One suggested that only the fully processed 32kDa inhibin was present in maternal serum (Muttukrishna *et al* 1995) while the other described very little 32kDa inhibin but a

predominance of a 55-60kDa form (Khalil *et al* 1995). While no studies were performed as part of this thesis to address this issue directly, for example with affinity chromatography, data related in *Chapters Three, Four* and *Seven* will suggest that multiple inhibin forms exist in pregnancy sera, as in non-pregnancy and as reported by Khalil and colleagues (1995), and in other pregnancy compartments, contrary to the conclusions of Muttukrishna and her colleagues (1995).

While the presence of multiple forms of inhibin, and the existence of specific and non-specific binding proteins, has clearly complicated the understanding of inhibin biology, the lack of suitable methods of detection has also hampered progress. *Chapter Two* discusses these problems and describes in detail the new sensitive and specific assays developed by Professor Nigel Groome, generously made available by him for the studies related here. As described in that chapter, the development of the latest generation of inhibin, and activin, assays has made possible the basis for the studies described hereafter in *Chapters Three* to *Nine.*

1.4 Inhibins and reproduction

Since the focus of this thesis is inhibins in human pregnancy, only a brief summary of inhibins in general human reproduction is offered here with an obvious emphasis on inhibins, rather than activins, in pregnancy. Nonetheless, it is hoped that this limited introduction will afford a suitable appreciation of the importance of inhibins and activins in general and further details are available from more extensive and excellent recent reviews (de Jong 1988, Ying 1988, Baird and Smith 1993, Woodruff and Mather 1995, Halvorson and DeCherney 1996, Wallace and Healy 1996).

While it was from the testis that inhibin-like activity was first demonstrated (McCullagh 1932), and the testis is indeed the main source of inhibin in men (Ishida 1990), the understanding of the roles of inhibin and activin within the male gonad are much less well defined than they are within the female. Both the Sertoli and Leydig cells in the testis secrete inhibin (Steinberger and Steinberger 1976, Le Gac *et al* 1982, Risbridger *et al* 1989), as evidenced by protein immunolocalisation and mRNA *in situ* studies (Cuevas *et al* 1987, Roberts 1989), but it is likely that the Sertoli cells are the principal source (Maddocks and Sharpe 1989) secreting inhibin under gonadotrophin control, primarily through regulation of the inhibin α-subunit (Roberts *et al* 1989, Krummen *et al* 1989) rather than either of the inhibin β-subunits (Roberts *et al* 1989). Indeed, both gonadotrophins are required for normal levels of circulating inhibin in men (McLachlan *et al* 1989b) and a large number of clinical studies have confirmed

that FSH and LH have important physiological roles in the control of testicular inhibin secretion (Burger and Scott 1981, McLachlan *et al* 1988a, 1988b, Burger *et al* 1988, Burger *et al* 1990, Yamaguchi *et al* 1991, Wallace *et al* 1993). However, despite elegant and convincing animal studies (Abeyawardene and Plant 1989, Medhamurthy 1991), until very recently there was no direct evidence to confirm that inhibin had a physiological role in the suppression of FSH secretion, the basis of the McCullagh's prediction (1932).

In clinical situations where it might have been expected that inhibin levels would be altered, such as seminiferous tubular damage associated with elevated circulating FSH, serum immunoreactive inhibin levels were observed to remain within the normal range (de Kretser 1989), contrary to the expectation that inhibin levels would be decreased, thereby releasing FSH from its suppression. However, recent studies using inhibin specific assays have shown that inhibin-B is the physiologically important form of inhibin in men (Illingworth *et al* 1996, Anawalt *et al* 1996, Nachtigall *et al* 1996, Anderson *et al* 1997, Wallace *et al* 1997) and that levels of inhibin-B fall progressively as FSH levels increase following testicular damage (Wallace *et al* 1997). Interestingly, the latter study also showed that pro-αC inhibin levels increased with increasing FSH, consistent with FSH stimulation of inhibin α-subunit (Toebosch *et al* 1988, Roberts *et al* 1989, Hancock *et al* 1992) and the observation that immunostaining for the inhibin α-subunit is increased in infertile men (Bergh and Cajander 1990). It was therefore suggested that the unchanged immunoreactive inhibin levels observed in the presence of increased FSH levels reported in previous studies composite changes in both inhibin-B (decreasing) and free α-subunit (increasing) (Wallace *et al* 1997). In summary, inhibins have both important intratesticular functions, helping to orchestrate spermatogenesis, and probably in consort with testosterone, and possibly oestrogens, an important endocrine function controlling FSH secretion from the pituitary.

There are many similarities between the inhibin secretion in women and that in men. Like the testis, the ovary is the main, if not sole, source of circulating inhibin (Illingworth *et al* 1991), with secretion from the granulosa cells, or lutein cells in the corpus luteum, controlled by both gonadotrophins and various other factors (Tsonis *et al* 1988, Hillier *et al* 1991a, Findlay 1993, Hee *et al* 1993). Circulating immunoreactive inhibin levels change throughout the menstrual cycle (McLachlan *et al* 1987a), with inverse changes in FSH levels, and recent studies using specific inhibin assays have related a similar pattern for inhibin-A (Muttakrishna *et al* 1994, Groome *et al* 1994) with transient changes in inhibin-B during the follicular phase when FSH levels are falling (Groome *et al* 1996). A large number of animal and human *in vitro*

and *in vivo* studies have explored the roles of inhibins within the ovary (Ying *et al* 1986, Hsueh 1986, LaPolt *et al* 1989, Woodruff *et al* 1990, Hillier *et al* 1991b, Hillier 1991, Xiao and Findlay 1991, Xiao *et al* 1992, Hillier and Miro 1993, Findlay 1993) and together they suggest that inhibin and activin, probably in consort with follistatin, ensure that there is both a mechanism of selecting the dominant follicle, ready for ovulation, and of preventing premature luteinisation (Findlay 1993, Baird and Smith 1993). As in the male therefore, in the female inhibins appear to have important intra-gonadal paracrine/autocrine functions and a traditional endocrine role in the suppression of FSH secretion.

1.5 Inhibins and pregnancy

In addition to controlling gonadal function, inhibins would appear to have important roles in pregnancy and it is this arena that is explored in this thesis, through various simple observational studies.

After ovulation the corpus luteum secretes inhibin-A and it would appear to continue to do so into early pregnancy (Illingworth *et al* 1996). However, women without functional ovaries have similar immunoreactive inhibin levels in early pregnancy to normal women (McLachlan *et al* 1987, Lenton *et al* 1991, Santoro *et al* 1992) suggesting that the ovary is not a major source of inhibin beyond six weeks of pregnancy. Indeed, a number of studies, reviewed by Qu and Thomas (1995), have suggested that the placenta is the main source of inhibins in pregnancy, as predicted by the first studies of placental extracts (Healy *et al* 1988) and primary trophoblast cell cultures (Petraglia *et al* 1987).

The control of inhibin secretion from the human placenta has not been fully elucidated, and indeed is the subject of further on-going studies (*Chapter Ten*), but *in vitro*, human chorionic gonadotrophin (hCG) and gonadotrophin releasing hormone (GnRH) stimulate trophoblastic inhibin secretion (Petraglia *et al* 1987, Li *et al* 1994) and reciprocally, inhibin suppresses hCG secretion. This latter effect is gestation dependant since there is no inhibin-induced hCG suppression in cell cultures derived from first trimester trophoblast (Mersol-Barg *et al* 1990). Opposing inhibin, activin stimulates hCG secretion from trophoblast cultures (Steele *et al* 1993) and inhibin and activin may both regulate progesterone and prostaglandin production (Petraglia *et al* 1993). Messenger RNAs for the three inhibin subunits, and the proteins themselves, have been localised to the syncytio- and cytotrophoblast (Petraglia *et al* 1987, Petraglia *et al* 1991, Minami *et al* 1992, Rabinovici *et al* 1992), the same cells secreting GnRH

and hCG (Petraglia *et al* 1992), with interesting changes in expression across gestations. In early pregnancy mRNA for the α-subunit is expressed in larger amounts than β_A-subunit mRNA, with very little β_B-subunit mRNA apparent (Petraglia *et al* 1991). With increasing gestation progressively more β_B-subunit mRNA is expressed suggesting that there is a gestation dependent switch from a predominance of inhibin-A and activin-A secretion to the presence of many forms (see below).

Circulating inhibin levels are significantly higher during pregnancy than in non-pregnancy (McLachlan *et al* 1987, Abe *et al* 1990, Tovanabutra *et al* 1993, Kettel *et al* 1991). Plasma immunoreactive inhibin levels peak at 9 -10 weeks, coinciding with the first trimester peak of hCG (Tovanabutra *et al* 1993), and then fall to a plateau between 15 and 30 weeks, rising thereafter so that the highest levels are achieved by term (Abe *et al* 1990, Tabei *et al* 1991, Qu *et al* 1991, Tovanabutra *et al* 1993). After delivery inhibin levels become undetectable within 4 - 5 days (Abe *et al* 1990, Kettel *et al* 1991, Qu and Thomas 1992, Tovanabutra *et al* 1993). Interestingly, bioactive inhibin levels have been reported to rise steadily throughout pregnancy without the biphasic profile described for immunoreactive inhibin (Qu *et al* 1991), consistent with a relative excess of inactive forms, either large molecular weight dimers or, more likely, free α-subunits, in early pregnancy, mirroring the relative excess of α-subunit message at this gestation (Petraglia *et al* 1991). However, using a specific assay, it was reported recently that maternal serum inhibin-A levels display a biphasic profile (Muttukrishna *et al* 1995) indicating that the bioassay data were probably confounded by inadequate stripping of the very high levels of circulating sex steroids in the test samples or by activins or other proteins that alter FSH secretion from the cells in culture. These issues are explored further in *Chapter Three*. In support of the placental *in situ* and immunolocalisation studies describing the ontogeny of subunit expression, inhibin-B is undetectable in peripheral serum during pregnancy (Illingworth *et al* 1996), a finding that is also re-examined and discussed in *Chapter Three*.

Activins have also been detected in the human placenta (de Kretser *et al* 1994, Knight *et al* 1996) and in maternal serum (Knight *et al* 1995, Petraglia *et al* 1995, Woodruff *et al* 1997), with a number of pieces of evidence to suggest that activins may have an important role to play in the onset of parturition (Petraglia *et al* 1993, Petraglia *et al* 1994a,b, Petraglia *et al* 1995, Woodruff *et al* 1997). Given the focus of this thesis however, no further consideration of these members of the inhibin family is given here.

In addition to examining the ontogeny of inhibins in normal pregnancy, a number of studies have recently assessed circulating levels of inhibin in a variety of pregnancy abnormalities. In pregnancies where it might be expected that placental mass would be reduced compared to normal, such as anembryonic pregnancies, ectopic pregnancies and threatened abortions, maternal serum inhibin levels, particularly proα-C immunoreactivity, are lower than in normal pregnancy (Yohkaichiya *et al* 1993, Illingworth *et al* 1996). It is therefore possible that inhibin may be useful in monitoring high risk first trimester pregnancies, although no studies have yet usefully assessed this possibility. Immunoreactive inhibin levels are higher in multiple pregnancies than in singletons (Yohkaichiya *et al* 1993, Khalil *et al* 1995) and data exploring this observation for inhibin-A are related in *Chapter Three*. Inhibin levels are also significantly higher than normal in association with various pregnancies complications such as pre-eclampsia, fetal growth restriction, or placental abruption (Khalil *et al* 1995). These complications are all associated with disruption of the trophoblast basement membrane and the higher serum inhibin levels may simply reflect leakage. However, inhibins apart, there is other evidence of placental endocrine dysfunction in such pregnancies, even in very early pregnancy (Gonen *et al* 1992) and there is a real prospect that maternal serum inhibin, with or without other markers,, may provide a basis for pregnancy complication screening. To date however, there has been no prospective assessment of any such predictive value of inhibin.

Inhibin is also markedly elevated in molar pregnancy (Yohkaichiya *et al* 1989) with an immunohistochemical study localising the inhibin subunits in molar trophoblast (Minami *et al* 1993) and suggesting that activin and inhibin may have roles in molar pathogenesis. However, it would appear that in a clinical setting, inhibin has nothing useful to add to hCG as a surveillance marker (Baddonel *et al* 1994)

Despite the focus of the aforementioned studies on the placenta as the source of inhibins in pregnancy, the studies and results detailed in *Chapter Three* explore other possible sources, reappraising current understanding and developing a basis from which to explore the control of inhibin secretion from different pregnancy tissues. In particular, the studies provide the basis from which inhibin secretion in Down's syndrome pregnancies can be better understood (see *Chapters Four*, *Five*, *Six* and *Eight*).

1.6 Down's syndrome: screening and diagnosis

It is not the intention to give an historical review of Down's syndrome but rather to focus on prenatal screening and diagnosis of the condition. However, detailed, and most interesting, accounts of the history of Down's syndrome as a clinical condition are given elsewhere (Smith and Berg 1976, Zellwerger 1977). Although the first medical illustration of an individual with Down's syndrome was in 1876 (Fraser and Mitchell 1876), much earlier representations may exist, including a fifteenth century painting of Madonna and child by Andrea Mantegna (Ruhrah 1935) and a seventeenth century painting by Jacob Jordaens (Zellwerger 1968). Further, while John Langdon Down is generally credited (Smith and Berg 1976) with the "discovery" in 1866 of a discrete clinical entity (Down 1866), which now carries his name, prior examples may have been reported (Esquirol 1838, Seguin 1846, Seguin 1866). In any event, the physical description of Down's syndrome was essentially complete by the beginning of this century (Smith and Berg 1976, Zellwerger 1977) and it was in 1959 that LeJeune and colleagues first identified that an extra chromosome 21 was the cause and Niebhur (1974) who suggested that gene replication was implied by this additional chromosomal material.

With an approximate incidence of 1.3 per 1000 live births, Down's syndrome is the single most important known cause of severe mental handicap in the developed world (Hook 1994). It is not surprising therefore that screening for Down's syndrome has become an important component of modern obstetric care (RCOG 1993), both for the expectant family who do not wish a Down's syndrome child and for society at large which finds screening and targeted termination highly cost effective (Gill *et al* 1987). It has long been recognised that an individual's risk of having an affected child increased with her age (Fraser and Mitchell 1876), with detailed risk estimates first calculated in five year intervals (Penrose and Smith 1966), forming the first basis of a screening test. The application of this screening test was only possible however with the availability of a diagnostic test for those women who were screen positive (ie older than 35 at the time of delivery). Such a diagnostic test became available with the successful karyotyping of fetal cells from amniotic fluid in 1966 (Steele and Breg 1967, Thiede *et al* 1967) and in particular with the description of second trimester amniocentesis the following year (Jacobson and Barter 1967) and the first diagnosis of Down's syndrome (Valenti *et al* 1968). Thus, using a mother's age as a screening test and an arbitrary cut-off age of 35, approximately 30% of Down's syndrome pregnancies will be detected (RCOG 1993).

However, almost all unexpected newborn babies with Down's syndrome are diagnosed morphologically and it has been long recognised that a number of congenital abnormalities, including cardiac anomalies (Garrod 1899) are common in the Down's syndrome fetus. Indeed, approximately 50% of individuals with Down's syndrome will have a congenital cardiac abnormality (Fabia and Drolette 1970) and the use of obstetric ultrasound affords the opportunity to detect many of these abnormalities prenatally. It is now recognised that a number of different abnormalities evident on ultrasound are associated with Down's syndrome and that the identification of any of these in a given pregnancy merits the offer of a diagnostic procedure. Such defects include duodenal atresia, cystic hygroma, nuchal oedema, pyelectasis, echogenic bowel, macroglossia, fifth digit middle phalanx hyoplasia and multiple combinations (Benacerraf 1985a,b, 1988, 1990, Nyberg *et al* 1990, Nicolaides 1993). However, many (63% in one series) of the abnormalities evident on ultrasound are transient (Bronshtein and Blumenfeld 1994) and are not apparent beyond 18 weeks, the traditional timing for a detailed fetal anomaly scan. Thus, ultrasound at 18-20 weeks will detect only 40% of Down's syndrome fetuses, approximately comparable with maternal age screening, while earlier examination may detect over 80% of affected fetuses (Nicolaides *et al* 1992, Nicolaides *et al* 1994, Bronshtein and Blumenfeld 1994).

In 1984 the observation that, on average, maternal serum levels of alpha-fetoprotein (AFP) were lower than normal in association with Down's syndrome (Merkatz *et al* 1984) represented the first significant progress in prenatal screening for Down's syndrome since the introduction of maternal age as a "screening test". Maternal serum AFP had long been used as a screening test for neural tube defects (Brock and Sutcliffe 1972), conditions where AFP levels are higher in than normal pregnancy. However, the finding of Merkatz and his colleagues (1984) offered the exciting possibility that other fetoplacental products may be useful markers of Down's syndrome or other aneuploidies (Chard *et al* 1984). This suggestion was realised with the subsequent report that human chorionic gonadotrophin (hCG) levels in maternal blood are elevated in association with Down's syndrome (Bogart *et al* 1987). A very large number of studies have confirmed this observation (for example Wald *et al* 1988, Osathanondh *et al* 1989, Suchy and Yeager 1990, Bogart *et al* 1991, Crossley *et al* 1991, Ryall *et al* 1992, Spencer *et al* 1992) and hCG, or more recently the free β-subunit of hCG (Macri *et al* 1990, 1993,1994, Spencer *et al* 1992, Ryall *et al* 1992), has become the single most powerful prenatal serum marker for Down's syndrome. Many prospective studies have now confirmed that screening programmes based upon

maternal age and the measurement of AFP and hCG (or free β-hCG), with or without other markers such as oestriol (Canick *et al* 1988, Wald *et al* 1988) or free α-hCG (Ryall *et al* 1992), affords an overall detection rate of 60-70% for an amniocentesis rate, or approximate false positive rate (FPR) of 5% (RCOG 1993). Such programmes are now widely available throughout the United Kingdom (RCOG 1993, Seth and Ellis 1994) and elsewhere (Ryall *et al* 1992, Golbus *et al* 1994, Mantingh *et al* 1994, Muller and Boué 1994).

However, it remains desirable to improve upon these detection rates, while minimising the FPR and the studies in *Chapter Five* relate the application of inhibin as a marker in the second trimester both alone and in combination with other markers. Furthermore, with the successful karyotyping from first trimester placental tissue by transcervical (Old *et al* 1982, Ward *et al* 1983, Simoni *et al* 1983) and then transabdominal (Smidt-Jensen *et al* 1984) chorion villus sampling diagnosis earlier than that afforded by second trimester amniocentesis became possible, thereby creating the desire for earlier screening. As discussed in more detail in *Chapters Four* and *Six*, a number of serum markers have been assessed in the first trimester (Macintosh and Chard 1993) and predicted detection rates look very promising indeed (Krantz *et al* 1996). Similarly, as discussed above and in *Chapter Six*, the use of ultrasound markers in the late first trimester looks extremely exciting. The studies in *Chapters Four* and *Six* describe the application of inhibin as a prenatal marker of Down's syndrome in the first trimester of pregnancy and relate this application to other biochemical and ultrasound markers at these early gestations, discussing the benefits and disadvantages of screening at this early gestation.

1.7 Conclusions

It is hoped that this chapter has afforded a useful background to both the biology of inhibins in reproduction and to the prenatal screening and diagnosis of Down's syndrome. The chapters that follow will expand upon this introduction, detailing a number of different studies reporting some novel observations in both inhibin biology and Down's syndrome screening. The last chapter will briefly review these data and more importantly, offer up for consideration the exciting future prospects that research in inhibins in human pregnancy may offer.

Chapter Two

Assay Methodology and Validation

2.1 Introduction

Prior to the development of radioimmunoassay, it was only possible to assess inhibin-like activity by observing changes in gonadal weight, reflecting FSH stimulation, under test conditions (Steelman and Pohley 1953). Subsequently, the recognition of the existence of a specific regulator of FSH secretion from the anterior pituitary gland (Franchimont *et al* 1972, Setchell *et al* 1974, Keogh *et al* 1974) and the development of a radioimmunoassay for FSH (Midgley 1967) afforded the development of other *in vivo* bioassays utilising the *in vivo* suppression of FSH in castrated animals (de Jong and Sharpe 1974, Marder *et al* 1977). However, the first sensitive and reliable assays for inhibin were *in vitro* bioassays using dispersed anterior pituitary cells (Vale *et al* 1972). Inhibin was measured by assessing either the suppression of FSH secretion by pituitary cells into culture media (Eddie *et al* 1979) or the decrease in pituitary cell content of FSH (Scott *et al* 1980). Unfortunately, the interpretation of results from such bioassays was complicated by the presence in the test sample of activins, binding proteins and other FSH-modulating substances which could oppose the *in vitro* actions of inhibin. Therefore, in reality the resulting "inhibin" levels were a crude balance between inhibin and activin bioactivity. Other bioassays utilising the differential actions of inhibin and activin on human erythroid stem cell lines was also developed (Yu *et al* 1987, Schwall and Lai 1991) but they also reflected composite levels of the two proteins and other interfering substances.

The purification of inhibin in 1985 (Robertson *et al* 1985, Ling *et al* 1985) and activin in 1986 (Vale *et al* 1986) however, allowed the generation of antibodies raised against either native protein or synthetic peptides. A variety of assays were thereafter developed (McLachlan *et al* 1986b, McLachlan *et al* 1987, Ying *et al* 1986, Hasegawa *et al* 1988, McNeilly *et al* 1988, Saito *et al* 1989, Vaughan *et al* 1989, Baly *et al* 1993), the most useful of which was a radioimmunoassay developed by McLachlan and co-workers, which became known as the "Monash assay", denoting its institution of origin, but the reagents for which were provided to the National Institutes of Health (NIH) for distribution worldwide.

This assay utilises a polyclonal antibody (AS 1989) raised against 31kD bovine inhibin with epitopes for the antibody on the inhibin α-subunit. Following its development, it became apparent that in addition to bioactive dimeric inhibin free inhibin α-subunits exist in serum (Knight *et al* 1989, Sugino *et al* 1989, Schneyer *et al* 1990) and that the Monash assay, and others using anti-inhibin α-subunit antibodies, detected these

(Schneyer *et al* 1990). As discussed in *Chapter One*, the free α-subunits are not thought to be biologically active and are of uncertain physiological relevance. Further, circulating binding proteins, such as follistatin and α_2-macroglobulin, were reported to alter antibody-antigen binding in clinical samples such as plasma (Vaughan and Vale 1993, Krummen *et al* 1993) and apparent inhibin levels were altered by *in vitro* protease activity (Bramley *et al* 1992). It is clear therefore that the interpretation of "inhibin" results obtained with α-subunit assays had to be necessarily cautious and limited (Burger 1993).

Recently, a new generation of two-site assays has been developed for both inhibins and activins, including assays specific for inhibin-A, inhibin-B, activin-A, activin-B and α-subunit precursors (Groome 1991, Knight *et al* 1991, Baly *et al* 1993, Woodruff *et al* 1993, Knight and Muttukrishna 1994, Woodruff *et al* 1994, Groome and O'Brien 1994, Poncelet and Franchimont 1994, Groome *et al* 1995, Knight *et al* 1996). These assays promise to clarify what were a number of apparent inconsistencies in data obtained with α-subunit based assays, exploring for the first time the control and relative ontogeny of the two mature forms of inhibin (inhibin-A and inhibin-B) and, by using a panel of assays, assessing levels of non-bioactive subunits of different molecular weights, exploring the biological processing of these proteins.

This thesis is concerned with the application of three sensitive inhibin enzyme-linked immunosorbent assays (ELISAs) specific for inhibin-A (Groome and O'Brien 1993), inhibin-B (Groome *et al* 1996) and pro-αC containing inhibins (Groome *et al* 1995) to explore the biology of inhibin in normal human and Down's syndrome pregnancy. In addition, a small study of immunoreactive inhibins, using two different immunoassays, in Down's syndrome pregnancies will be reported.

2.2 Immunoreactive inhibin assays

In *Chapter Four* a small preliminary study is reported using two different inhibin immunoassays. The methodology of these are described here.

2.2.1 "Monash" inhibin assay.

This assay is a heterologous radioimmunoassay using an antibody (AS 1989) raised against 31kDa bovine inhibin and a tracer of iodinated 31kDa bovine inhibin (McLachlan *et al* 1986, McLachlan *et al* 1987). Recombinant human inhibin A (rhINH-R-90/1) was used for standards with the results expressed as pg/ml. The

sensitivity of the assay was 780pg/ml and the coefficients of variation of intra-assay and inter-assay were 6.6% and 11.5% respectively. This assay is now distributed by the National Institute of Child Health and Human Development (NICHD), a section of NIH.

2.2.1.1 Materials required

1. Assay buffer: 0.01M PBS, 0.5% BSA (ph7.4)
2. Tracer buffer: Assay buffer +0.1% Triton X
3. 1st antibody: 1in 3000 antisera (AS#1989) (NICHD) and
   1in 400 normal rabbit serum (#5162L) (SAPU, Scotland, UK)
4. 2nd antibody: 1in 64 donkey anti-rabbit serum (#5257M) (SAPU, Scotland, UK)
5. Tracer: ^{125}I bovine 31K Inhibin (iodinated in house) (NICHD)
6. Standards: purified human follicular fluid, calibrated against ovine rete testis fluid in a sheep pituitary bioassay (in-house).

2.2.1.2 Methodology

1. Prepare buffer, 1st antibody and standards (from storage).
2. Add sample (200μl or 300μl), or standard (100μl) and 100μl 1st antibody (± buffer depending on standards used) to each LP3 tube and vortex. See method grids below for volumes.
3. Incubate at room temperature for 24 hours.
4. Prepare tracer buffer. To make tracer solution add approximately 100μl of stock tracer to 10mls of tracer buffer. Adjust to allow for ~10,000 cpm in 100μl of tracer.
5. Add 100μl tracer to all tubes and cap Tc tubes. Vortex. Incubate at room temperature for 24 hours.
6. Prepare 2nd antibody and add 100μl to all tubes except Tcs. Vortex. Incubate at 4°C overnight (16 hours).
7. Prepare normal saline and add 2mls to each tube except Tc's.
8. Centrifuge at 2500rpm for 45mins at 4°C.
9. Aspirate the supernatant carefully and keep the tubes upright prior to counting. In large assays keep tubes at 4°C while waiting to aspirate. Counting can be performed immediately.

Method grid for 200µl sample and standards in buffer.

sample/volume	Buffer	Antibody	Tracer	STD/Sample	Serum
Tc	-	-	100	-	
NSB	200	-	100	-	200
Bo	100	100	100	-	200
STDS	-	100	100	100	200
Sample	100	100	100	200	-

Method grid for 300µl sample and standards in serum

sample/volume	Buffer	Antibody	Tracer	STD/Sample	Serum
Tc	-	-	100	-	-
NSB	100	-	100	-	300
Bo	-	100	100	-	300
STDS	-	100	100	100	200
Sample	-	100	100	300	-

Method grid for 300µl sample and standards in buffer

sample/volume	Buffer	Antibody	Tracer	STD/Sample	Serum
Tc	-	-	100	-	-
NSB	200	-	100	-	300
Bo	100	100	100	-	300
STDS	-	100	100	100	300
Sample	100	100	100	300	-

2.2.1.3 Assay buffers

Three buffer solutions are required for the Monash based RIA for human Inhibin:

1. Assay buffer: 0.01M PBS, 0.5% BSA, pH7.4

i. Make stock solution (0.5M PBS) by:
Add 7.8g $NaH_2PO_4.2H_2O$ (MWt = 156.01g) to 100mls distilled water (0.5M sol^n)
Add 7.1g of Na_2HPO_4 andydrous (MWt = 141.96g) to 100mls distilled water (0.5M sol^n). Mix 23.5mls of the first solution with 100mls of the second and adjust pH to 7.4. Store at -20°C in 10ml aliquots.

ii. Dilute stock (0.5MPBS) 1in 50 by adding 490mls distilled water to a 10ml aliquot

iii. Add 4.5g NaCl, 500mg Na Azide, 2.5g BSA

2. Tracer buffer:

i. Add 0.1% (v/v) Triton X to Assay buffer.

3. Separation stage buffer: normal saline (0.9% NaCl)

2.2.2 Medgenix assay

The second immunoassay used was a commercial solid-phase two site immunoenzymatic assay (Medgenix, High Wycombe UK), used according to the manufacturers instructions. The two antibodies employed in this assay were directed against distinct epitopes of the α-subunit of human inhibin. Human inhibin was used as standards and the results were expressed as U/ml. The sensitivity of the assay was 0.1U/ml and the coefficients of variation of intra-assay and inter-assay were 1.9% and 8.9% respectively.

There are reports of the use of both of the inhibin immunoassays in human sera in pregnancy (McLachlan *et al* 1987, Yohkaichiya *et al* 1991, Tabei *et al* 1991, Kettel *et al* 1991, van Lith *et al* 1992, Yohkaichiya *et al* 1993) and no further validation of these assays was performed.

2.3 Inhibin enzyme linked immunosorbent assays (ELISAs)

2.3.1 Inhibin-A ELISA

The preparation of monoclonal antibodies to the inhibin α-subunit (Groome *et al* 1990) and subsequently the inhibin β-subunit (Groome and Lawrence 1991) allowed the development of a two-site immunoassay for inhibin-A (Groome 1991). Assay sensitivity sufficient for the application of the assay to human sera was achieved with modifications to the assay format (Groome and O'Brien 1993), including a pre-assay sample treatment step that oxidises the methionine residues in the β-subunit epitope

(Knight and Muttukrishna 1994). The assay has been previously validated for human sera, firstly in non-pregnant menstrual cycle sera (Groome *et al* 1994, Muttukrishna *et al* 1994) and then in pregnancy sera (Muttukrishna *et al* 1995).

During the course of the studies undertaken for this thesis a number of further modifications were made to this ELISA. These modifications were suggested by Nigel Groome and led to significant cost savings and improved assay performance. The original method (format I) and the various modifications are described succinctly below and simply referred to by format number elsewhere in the thesis. The current, preferred format is format III (see also *Chapter Seven*) and the full methodology of this format is given in detail.

Format I: Prior to adding to the microtitre plate, each sample or standard is mixed with 1% hydrogen peroxide (final w/v) and incubated at room temperature for 30 minutes. This is the peroxide step that modifies the ß-subunit epitopes to improve reactivity with the respective antibody (Knight and Muttukrishna 1994) and thereby increases the assay sensitivity. Each sample or standard is then diluted 1:1 in assay diluent (0.1M Tris HCl, 0.15M NaCl with 5% triton X100, 10% bovine serum albumin, 5% normal mouse serum, pH7.5) and then added to the plate. The assay utilises an immobilised anti β_A-inhibin subunit monoclonal antibody (E4) as a capture antibody, covalently coupled to hydrazide microplates (Avidplate-HZ, UniSyn Technologies, CA, USA). The Fab fraction of a mouse anti α-inhibin subunit monoclonal antibody (R1) is used as a second antibody diluted in assay diluent. This is conjugated to alkaline phosphatase, allowing detection by the addition of an amplified enzyme assay (Life Technologies, Paisley, UK) which affords colour change. Absorbency is measured after 20-30 minutes at 490nm in a microplate reader (Thermomax, Molecular Devices Corp., CA, USA) using dedicated software (Softmax, Molecular Devices Corp.). Results are expressed as pg/ml. The sensitivity of the assay is 8 pg/ml and the intra- and inter-plate coefficients of variation are 4.7% and 10%, respectively.

Format II - This method is identical to method I except for the method of signal detection. The high degree of sensitivity developed in the original method, while required for the detection of inhibin-A throughout the human menstrual cycle (Groome *et al* 1994), was not required for the very high levels of inhibin-A in pregnancy sera (*Chapters Four, Five* and *Six*). A simplified and cheaper method of detection using a non-amplified alkaline phosphatase substrate, p-nitrophenylphosphate (pNPP), (Kirkegaard and Perry Laboratories, Maryland, USA) was therefore validated by Groome. Using this method plates are read after 2-4hrs at 405 nm in a microplate

reader as before. Results are expressed as pg/ml with an assay sensitivity of 23 pg/ml. The intraplate and interplate coefficients of variation are 5.6% and 6.3%, respectively.

Format III - This method involves an additional sample preparation step, performed prior to oxidation. During the early application of the assay to clinical serum samples it was observed that signals could not be obtained from some samples, particularly, though not exclusively, those with evidence of haemolysis (E Wallace, personal observation). This was believed to be due to a reaction between catalase, an enzyme released by red blood cells on lysing, and the peroxide, quenching the oxidative effect on the inhibin. Recently, Groome and his colleagues developed and described ELISAs similar to the inhibin-A assay but specific for inhibin-B and activin-A (Groome *et al* 1996, Knight *et al* 1996). These latter assays incorporated a pretreatment step, involving heating the sample or standard in a waterbath at 100°C for 3 minutes after mixing with 2% (final w/v) sodium dodecylsulphate (SDS). This was found to be effective in removing interfering substances from the samples and was subsequently applied to the inhibin-A assay. While parallelism, recovery and sensitivity of the modified assay are comparable to the original method (Groome, in-house data) it was observed that with the modification signals could be generated from all samples, including those in which no inhibin had been previously detectable. Detection is afforded by the addition of pNPP as detailed in method II. Results are expressed as pg/ml with an assay sensitivity of 23 pg/ml. The intra- and inter-plate co-efficient of variation are 4.9% and 8.7%, respectively.

Standard preparations: in addition to the modifications to the assay format described above, Nigel Groome prepared an in-house calibrator, allowing the commercial distribution of his reagents. Some of the studies reported in this thesis utilised the original standard (a 32kD recombinant human (rh) inhibin supplied by Genentech Inc.,CA, USA), denoted in this thesis by "RH", while others utilised the new preparation. This is partially purified from human follicular fluid and was calibrated against the rh inhibin using assay format I. It is denoted by "IP" hereafter.

The full methodology of the inhibin-A ELISA (format III) is:

2.3.1.1 Oxidation of primary antibody, E_4 (anti- inhibin β-subunit Ab)

1. Take 2 mls of E_4 Ab (1mg/mL), out of the refrigerator.
2. Take the benzoylated dialysis tubing out of the refrigerator, cut an appropriate piece off and wash with distilled water.
3. Put the 2 mls E_4 Ab in the dialysis tubing and close the tubing securely with clips.
4. Dialyse E_4 Ab against 3 changes of 5 litres 50mM acetate buffer (sol^n 1) at 4°C.
5. Add 0.1 volume sodium periodate solution (sol^n 2) to the E_4 Ab and incubate in the dark at room temperature.
6. Add 0.01 volume of ethylene glycol.
7. Dialyse the antibody solution against 5 litres of acetate buffer at 4°C, overnight.
8. Read the final absorbance in a 1 cm cell, to calculate amount of antibody. Ab concentration (mb/mL) = absorbance/1.4.

2.3.1.2 Coating plates

1. Remove dry plates (Avidplate-HZ, UniSyn Technologies, CA, USA) from refrigerator. Bring to room temperature.
2. Make up Ab/coating solution (sol^n 3), using stock coating solution (sol^n 2) and oxidised E_4 Ab.
3. Add 50μl solution to each well. Incubate at room temperature in a covered box, keeping moist, overnight.
4. One plate at a time, bang dry and add 150μl of blocking solution (sol^n 4).
5. Plates can be stored refrigerated for 3-6 months. Evaporation must be prevented.

2.3.1.3 Assay method

1. Defrost samples and standards. Mix on vortex when thawed.
2. Defrost assay diluent (sol^n 5).
3. If the expected inhibin level is in excess of top standard (1500pg/ml) then dilute appropriately with fetal calf serum.
4. Put 100μl of each standard and bloodsample, or 50μl of amniotic fluid sample into a 2mL Sarstedt tube.
5. Add 50μl of 6% SDS (sol^n 6) to each standard and sample. Mix.
6. Using polystyrene "floats" place all standards and samples in boiling water for 3 minutes. Allow to cool.
7. Add 10μl of 15% hydrogen peroxide (sol^n 7) to each sample or standard. Mix. Allow to stand for 30 minutes.
8. Remove plates from refrigerator.
9. Add 100μl of assay diluent to each tube. Mix.

10. Take an E_4 coated hydrazide plate and wash it 10 times in wash buffer (sol^n 8). Bang dry on tissue. Without allowing the plate to dry, add 100μl of the sample to duplicate wells on the microtitre plate. Cover the plate with a seal and place in the refrigerator.
11. Incubate the plate overnight. Shaking is not required.
12. The following day, wash the plate 4 times with wash buffer and bang dry.
13. Immediately add 50μl Fab R1 alkaline phosphatase conjugate to each well (sol^n 9). Reseal plate and shake at room temperature for 1 to 2 hours.
14. Wash the plate 10 times in wash buffer and bang dry.
15. Immediately add 100μl of alkaline phosphatase substrate (pNPP) (sol^n 10). Reseal the plate and shake for 3 to 4 hours.
16. Stop the colour change with 100μl of 5% EDTA (sol^n 11). Read the plate with plate reader at 405nm and 650nm.

2.3.1.4 Assay solutions

1. Acetate buffer / Coating solution: Add 20.5g Na acetate-anhydrous and 42.5g NaCl to 5000mls of distilled H_2O. pH was adjusted to 5.0.
2. Sodium periodate solution (100mM): Add 21mg Na periodate to 1ml distilled H_2O.
3. Antibody coating solution (5μg Ab /ml): To coat 20 plates 100mls (500 μg Ab) are required. Add Ab to Sol^n 1.
4. Blocking solution: Add 100mls stock 0.4M HCl to 300mls distilled H_2O (0.1M HCl) Discard 50mls and add 4.24g Tris (1.22g/100mls), 3.5g of bovine serum albumine and 0.35g sodium azide. Adjust pH to 7.5
5. Assay diluent: Add 12.10g Tris and 8.8g of NaCl to 1000mls 0.1M HCl. Adjust pH to 7.5 with NaOH. Remove 100mls and add 50mls of triton X100 (5% w/v), 100g protease free bovine serum albumin (10% w/v), 50mls of normal mouse serum (pre-filtered 0.4μl filter) and 1g of NA sodium azide. Freeze in 100ml aliquots.
6. Sodium dodecyl sulfate solution: Add 6g of SDS to 100 mls distilled H_2O.
7. Hydrogen peroxide solution (15%): Add 3mL stock hydrogen peroxide (30%) to 3mL distilled H_2O.
8. Wash buffer solution: Add 52.62g NaCl, 36.36g Tris, and 3mls of Tween 20 to 5.5L distilled H_2O. Using HCl adjust to pH 7.5 and make up to 6L with distilled H_2O. Store at room temperature.
9. Fab R1 alkaline phosphatase conjugate (1:200 dil.): Add 120μl of R1 to 24mls sol^n 5

10. P-nitrophenylphosphate substrate: Add 9mls of diethanolamine concentrate, 9 substrate tablets and 45μl of 1M magnesium chloride to 36mls distilled H_2O.
11. 5% ethylenediaminetetra-acetic-acid solution: Add 5g EDTA disodium salt to 100mls distilled H2O.

2.3.2 Inhibin-B ELISA

Inhibin-B was measured using a similar two-site ELISA as previously described (Groome *et al* 1996). The detailed protocol and assay buffers and wash solution are essentially identical. A monoclonal antibody (C5) raised against the human inhibin β_B subunit was used as a capture antibody, biotinylated and immobilised on streptavidin-coated microplates (Life Sciences International, Basingstoke, UK). The same second antibody (R1) as used in the inhibin-A assay was employed. Samples and standards were also pretreated as detailed above, but in this assay the signal was detected using the alkaline phosphatase amplification kit detailed in format I of the inhibin-A assay (Life Technologies, Paisley, UK). Standard preparation IP was used as a standard but was calibrated against recombinant human inhibin-B with results expressed in pg/ml. Plates were read at 490nm using the same apparatus and software as detailed for the inhibin-A assay. The assay detection limit was less than 5pg/ml. Activin-A, activin-B, follistatin and purified human pro-αC had less than 0.1% cross reaction while recombinant inhibin-A had a 0.5% cross reaction. The intra- and inter-plate co-efficients of variation are 7.6% and 8.1%, respectively.

The full methodology of the inhibin-B ELISA is:

2.3.2.1 Coating Plates

1. Remove streptavidin coated dry plates (Combiplate 8) from refrigerator. Bring to room temperature.
2. Make up Ab/coating solution. (5μg/ml biotinylated C5 in 1% BSA Tris-HCl, pH 7.5)
3. One plate at a time, wash several times in distilled H_2O and add 50μl Ab solution to each well.
4. Incubate at room temperature in a covered box, keeping moist, overnight.
5. One plate at a time, bang dry and add 100μl of blocking solution (Sol^{n} 4 above). Plates can be stored refrigerated for 3-6 months. Evaporation must be prevented.

2.3.2.2 Assay method

1. Defrost samples and standards. Mix on vortex when thawed.
2. Defrost assay diluent (sol^{n} 5).
3. If the expected inhibin level is in excess of top standard (1625pg/ml) then dilute appropriately with fetal calf serum.
4. Put 100μl of each standard and sample into a 2mL Sarstedt tube.
5. Add 50μl of 6% SDS (sol^{n} 6) to each standard and sample. Mix.
6. Using polystyrene "floats" place all standards and samples in boiling water for 3 minutes. Allow to cool.
7. Add 10μl of 15% hydrogen peroxide (sol^{n} 7) to each sample or standard. Mix. Allow to stand for 30 minutes.
8. Remove plates from refrigerator.
9. Add 100μl of assay diluent to each tube. Mix.
10. Take an C5 coated streptavidin plate and wash it 10 times in wash buffer (sol^{n} 8). Bang dry on tissue. Without allowing the plate to dry, add 100μl of the sample to duplicate wells on the micro plate. Cover the plate with a seal and place in the refrigerator.
11. Incubate the plate overnight. Shaking is not required.
12. The following day, wash the plate 10 times with wash buffer and bang dry.
13. Immediately add 50μl of the Fab R1 alkaline phosphatase conjugate (sol^{n} 9) to each well. Reseal plate and shake at room temperature for 1 to 2 hours.
14. Reconstitute alkaline phosphatase substrate (2 bottles for 4 plates). Mix, stand for 10 minutes prior to use. (Remember to add 12μl MgCl to substrate)
15. Wash the plate 10 times in wash buffer and bang dry.
16. Immediately add 50μl of alkaline phosphatase substrate. Reseal the plate and incubate at 37°C for 2 hours.
17. Reconstitute alkaline phosphatase amplifier (2 bottles for 4 plates). Mix, stand for 10 minutes prior to use.
18. Add 50μl of amplifier to each well. Watch as colour develops and stop with 50μl of 0.4M HCl. Read plate at 490nm and 650nm.

2.3.3 Pro-αC containing inhibins ELISA

Inhibin forms containing pro-αC were detected using the specific ELISA as recently described (Groome *et al.*, 1995) with some modifications. This assay uses a capture monoclonal antibody (INPRO) raised against a sequence of the pro-αC α-subunit, and an immunopurified standard calibrated against rh 32kDa inhibin, as described in detail (Groome *et al* 1995). Unlike the inhibin-A and inhibin-B assays, after diluting samples

with assay buffer, standards and samples samples were added directly to the plate without pre-assay oxidation or boiling. The same detection antibody (R1) was used as in the dimeric inhibin assays, and the alkaline phosphatase activity was detected using pNPP substrate, as in the inhibin-A assay formats II and III. Recombinant forms of inhibin-A, inhibin-B and follistatin all cross-react less than 0.02%, although this antibody may cross-react with the larger dimeric inhibin isoforms containing the α-subunit pro sequences as demonstrated by immunoblotting studies (Groome *et al* 1995). The intra- and inter-plate coefficients of variation are 5.2% and 8.1%, respectively. The detection limit was 3 pg/ml.

The full methodology of the inhibin pro-αC ELISA is:

2.3.3.1 Assay method

1. Defrost samples and standards. Mix on vortex when thawed.
2. Remove plates from refrigerator.
3. If the expected inhibin level is in excess of top standard (100pg/ml) then dilute appropriately with fetal calf serum.
4. Take a Pro-αC coated plate and wash it 10 times in wash buffer. Bang dry on tissue. Without allowing the plate to dry, add 50μl of the sample / standard to duplicate wells on the microplate. Cover the plate with a seal and place in the refrigerator.
5. Incubate the plate overnight. Shaking is not required.
6. The following day, wash the plate 10 times with wash buffer and bang dry.
7. Immediately add 50μl of the Fab R1 alkaline phosphatase conjugate (check dilution - this may vary) to each well. Reseal plate and shake at room temperature for 1-2 hours.
8. Switch on incubator, set for 37°C, ready for step 11.
9. Reconstitute alkaline phosphatase substrate (2 bottles for 4 plates). Mix, stand for 10 minutes prior to use. (Remember to add 12μl MgCl to substrate)
10. Wash the plate 10 times in wash buffer and bang dry.
11. Immediately add 50μl of alkaline phosphatase substrate. Reseal the plate and incubate at 37°C for 2 hours.
12. Remove plates from incubator and allow to cool for 5 minutes.
13. Reconstitute alkaline phosphatase amplifier (2 bottles for 4 plates). Mix, stand for 10 minutes prior to use.
14. Add 50μl of amplifier to each well. Watch as colour develops and stop with 50μl of 0.4M HCl. Read plate at 490nm and 650nm.

2.4 Validation of the inhibin-A ELISA

The use of the inhibin-A, inhibin-B and pro-aC inhibins ELISAs in pregnancy sera has been previously reported and validated (Muttukrishna *et al* 1995, Illingworth *et al* 1996a). However, there are no previous studies reporting their application to human amniotic fluid or extra-embryonic coelomic fluid, nor are there studies describing the performance of the different inhibin-A ELISA formats. Validation of the inhibin-A ELISA in extra-embryonic coelomic fluid and the inhibin-B and pro-αC inhibin ELISAs in amniotic fluid and extra-embryonic coelomic fluid was performed by Dr Simon Riley, University of Edinburgh specifically for these studies. Details of these procedures are therefore not reported here, as is appropriate, but are reported elsewhere (Riley *et al* 1996, Wallace *et al* 1997). However, validation of the inhibin-A assay in amniotic fluid and formal assessment of the performance of this assay in clinical samples is reported here, representing original and novel work.

2.4.1 Serum stability study

The potential usefulness of any marker of Down's syndrome will depend in part upon the stability of the marker *in vitro*. For example, levels of free β-hCG increase significantly over 72 hours under conditions similar to those that would be expected during sample transport between doctor and laboratory (Sancken and Bahner 1995), an effect that may alter risk estimates and so require correction prior to interpretation. To explore the possibility that storage might alter the level of inhibin-A detected a small stability study was undertaken.

2.4.1.1 Methods

Blood was collected, with consent, by routine venepuncture from ten normal women in midpregnancy. Each sample was divided into two aliquots. One aliquot was centrifuged and the other kept unseparated at room temperature. The serum from the former aliquot was further divided into three sub-aliquots, one of which was frozen at -20°C immediately. The remaining two serum sub-aliquots were kept at room temperature with subsequent frozen storage (-20°C) of one after 24 hours and the other after 48 hours. The whole blood aliquot was similarly divided into 2 sub-aliquots. These were centrifuged at 24 and 48 hours, allowing frozen storage (-20°C) of the serum at those times. Inhibin-A levels were measured using assay formats II and III with the IP standard. Ethical approval was obtained from the Lothian Research Ethics Committee.

2.4.1.2 Statistical analyses

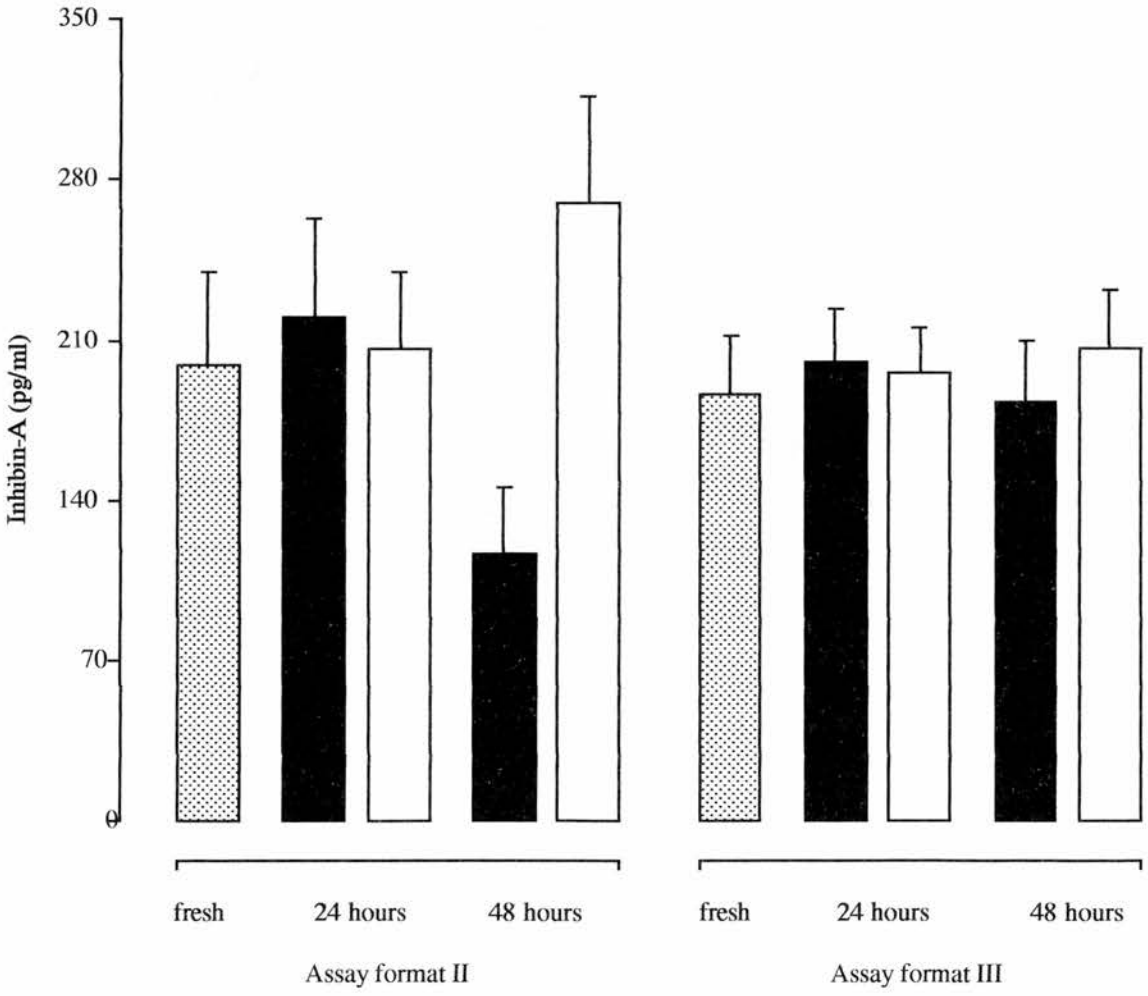
Statistical analyses were performed using the software package Statview 4.1 (Abacus Inc., Berkeley, CA, USA). Inhibin-A levelswere subjected to two-way analysis of variance (ANOVA) for repeated measures. Significance was recognised when $p<0.05$.

2.4.1.3 Results

Figure 2.1 shows the mean ± SEM inhibin-A level in the ten samples as measured by assay formats II and III and by method of storage. Using format II the mean inhibin-A level in samples stored as whole blood declined significantly ($p<0.01$), with a fall of 42% at 48 hours, whereas levels were stable for the samples stored as serum. When format III was employed however, no significant changes in the mean inhibin-A level were observed, regardless of how the samples had been stored.

Figure 2.1.

Changes in inhibin-A levels with storage (mean ±SEM) in samples from 10 pregnant women. Baseline sample = ⊠, whole blood= ■, serum= □.



2.4.2 Freeze-thaw stability study

Similarly, considering that many of the studies related in this thesis were performed using archival sera, some of which had been freeze-thawed on more than one occasion, it was considered important to examine whether these processes exerted a significant effect on inhibin-A levels.

2.4.2.1 Methods

Blood samples were collected, with written informed consent, from eleven women in midpregnancy. In each case, the serum was separated in to six aliquots. On the day of collection, the fresh sample was assayed, without freezing, for inhibin-A. Each of the other aliquots was exposed to from one to five freeze-thaw cycles and then assayed for inhibin-A.

2.4.2.2 Statistical analyses

Statistical analyses were performed using the software package Statview 4.1 (Abacus Inc., Berkeley, CA, USA). The levels of inhibin-A, expressed as a percentage of baseline, were subjected to two-way analysis of variance (ANOVA) for repeated measures. Significance was recognised when $p<0.05$.

2.4.2.3 Results

There was no significant change in inhibin-A levels with repetitive freeze-thawing of up to five cycles (figure 2.2, page 29).

2.4.3 Assessment of the effect of incubation time on inhibin-A levels using format II

In addition to affording significant cost benefits, the use of a simple alkaline phosphatase substrate in the inhibin-A ELISA format II, in place of the amplification kit in format I, allowed plate reading to be performed over a very broad time window. In comparison, using format I colour changes was often maximum at only 10-15 minutes after adding the amplifier. However, no data were available to assess the effects of prolonging the incubation time following the addition of the substrate pNPP nor to allow a comparison between this format and format I.

2.4.3.1 Methods

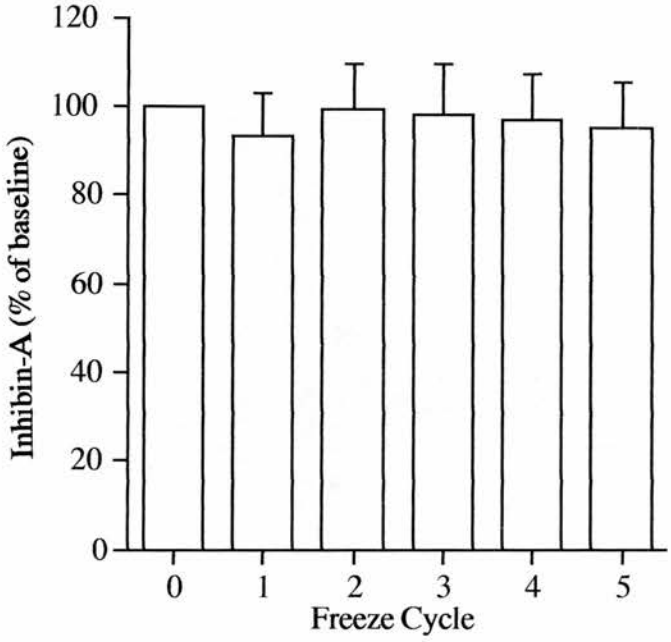
Two assays with identical samples were run simultaneously, one using format I and one using format II. No stop solution was added to the format II plate allowing multiple readings over several hours. Levels of inhibin-A were then compared between methods and over time.

2.4.3.2 Statistical analyses

Statistical analyses were performed using the software package Statview 4.1 (Abacus Inc., Berkeley, CA, USA). Simple regression analysis was performed between the results obtained by the two methods or between readings.

Figure 2.2

Inhibin-A levels (mean ±SD), expressed as a percentage of baseline (fresh) in 11 pregnancy sera during repetitive freeze-thawing.



2.4.3.3 Results

There was a strong and highly significant correlation of inhibin-A levels obtained by format I with format II, irrespective of when the format II plate was read (figure 2.3, page 30) . Similarly, delayed reading, up to 20 hours, did not affect the relative levels of inhibin-A obtained with format II, except that with increasing time the samples with high levels, and the top standards, became un-readable.

2.4.4 Validation of inhibin-A assay in amniotic fluid.

2.4.4.1 Methods

Recombinant human inhibin-A was added to ten amniotic fluid samples, each of known inhibin-A content. Serial dilution of amniotic fluid samples was also performed and the dilutions assayed for inhibin-A.

2.4.4.2 Results

Recovery of recombinant human inhibin-A spiked into AF was quantitative (mean ±SEM 108 ± 13%, n=10). Serial dilution of AF samples gave dose responses parallel to that of the immunopurified standard (figure 2.4, page 31).

Figure 2.3

Regression plots of inhibin-A levels obtained using ELISA formats I and II and of inhibin-A levels over time of plate incubation in format II.

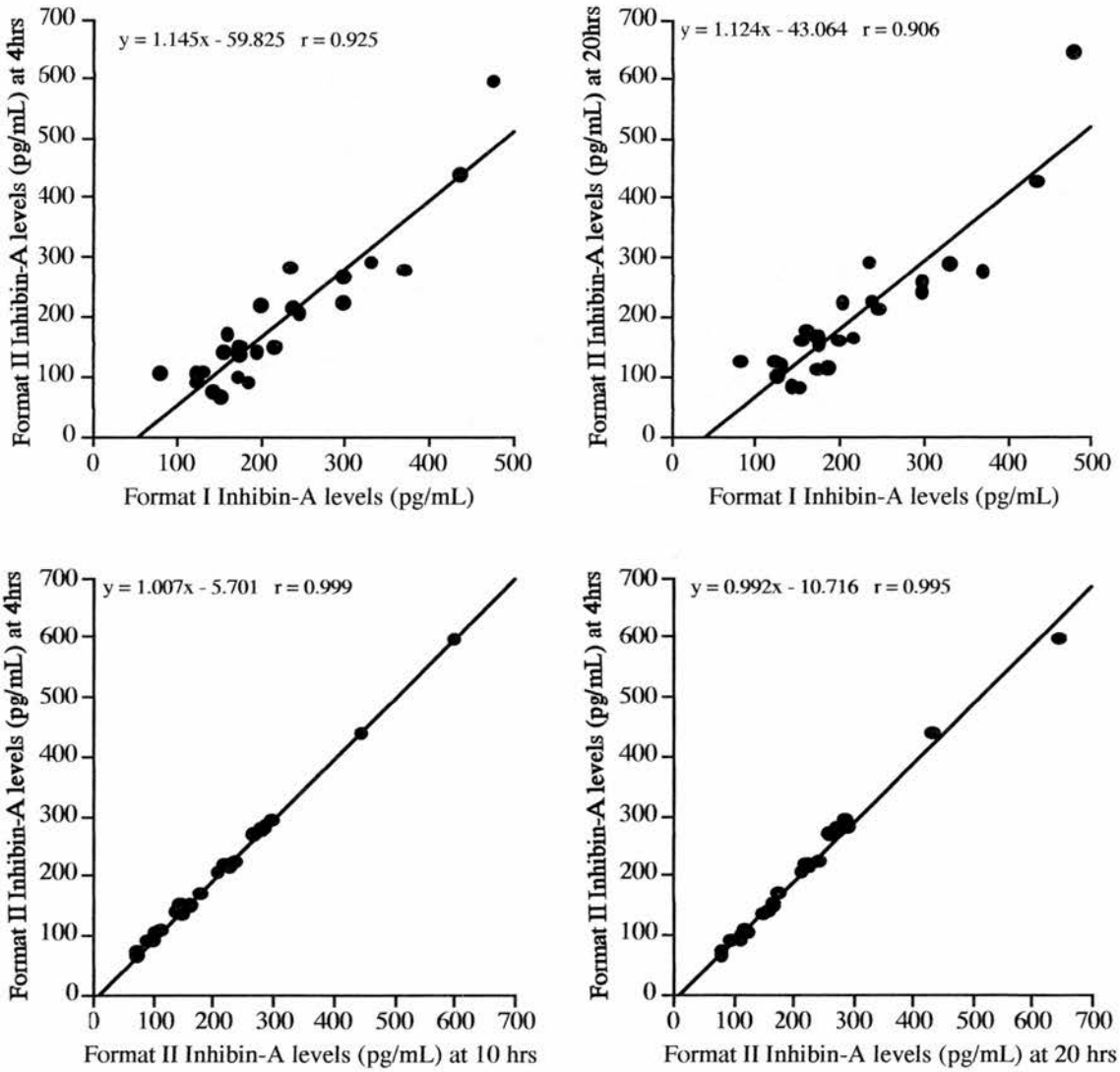
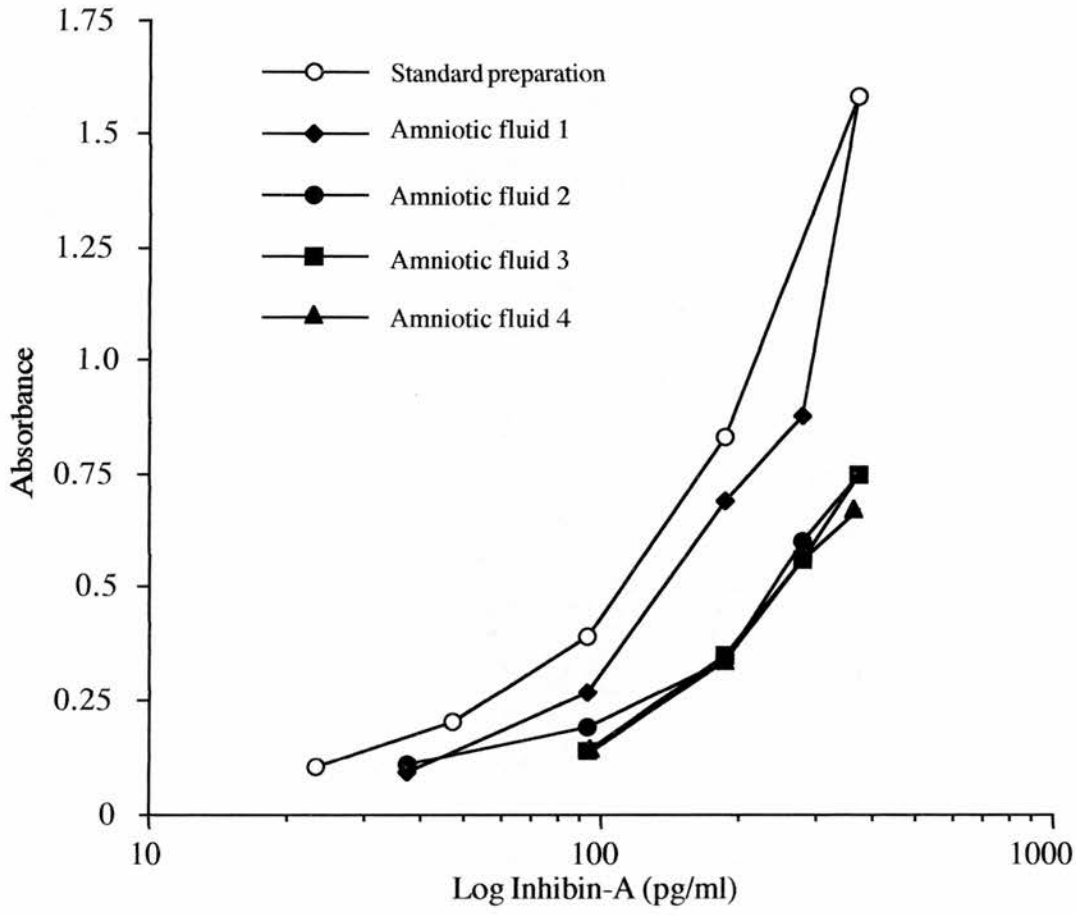


Figure 2.4

Dose-response relationships for immunopurified inhibin standards with serially diluted amniotic fluid samples in the inhibin-A ELISA.



2.5 Discussion and conclusions

This chapter has described in detail the different inhibin immunoassays used in the studies reported in this thesis. In particular, the recently developed sensitive and specific ELISAs for inhibin-A, inhibin-B and pro-αC inhibins are described in detail. Further, since the inhibin-A ELISA evolved considerably over the course of the studies, this process of evolution is described and evaluated. *Chapter Seven* re-visits this issue in the context of Down's syndrome screening and affords insights into, not only the assays themselves, but also into the circulating forms of inhibin that may exist in maternal serum.

The simple stability studies showed that inhibin-A is stable under repetitive freeze-thaw cycles to at least five cycles. Further, the studies showed that inhibin-A levels fell when samples were stored, at room temperature, as whole blood but not as serum, and that when the samples were boiled prior to assay the decline was prevented. The changes observed with format II (no boiling) could not have been due to dissociation of the inhibin dimer, because levels were stable with format III (SDS boiling), but rather to an effect related to storage as whole blood, but not serum. This is quite different to hCG which dissociates during storage (Sancken and Bahner 1995), an effect recently shown to be secondary to microbial action (Kardana and Cole 1997). It is therefore suggested, and discussed again in more detail in *Chapter* Seven, that this effect is due to the release of catalase from the red blood cells, which will quench the oxidising effect of the hydrogen peroxide pretreatment step. Importantly, a quenched inhibin-A signal was evident in the stability study samples even without obvious haemolysis. These data therefore suggest that while the apparent inhibin-A levels is stable in clinical samples, this is only so when using format III. In the context of possible prenatal screening programmes, when samples might travel for some distance and time prior to assay, this is clearly an important observation.

The introduction of a simple alkaline phosphatase substrate (formats II and III) afforded significant cost savings. However, the colour change reaction was much slower than that observed with the amplified substrate method used in format I and it was not known what the stability of this process was. The simple experiment detailed in *2.4.3* showed that not only were the results obtained with formats I and II very similar ($r<0.9$), a finding expanded upon in *Chapter Seven*, but that this was so, irrespective of when the format II plate was read, up to 20 hours. Similarly, levels of inhibin-A obtained with format II did not change with time of plate reading, again up to 20 hours. This was an important finding because it allowed many plates to be run consecutively and then read the following day.

The spiking and serial dilution experiments represent simple methods of assay validation for amniotic fluid. As in serum (Groome *et al* 1994), it would appear that the ELISA is detecting inhibin-A.

In conclusion, the series of small, but necessary, experiments related in this chapter have set the foundations on which the studies detailed in the following chapters are based.

Chapter Three

Inhibins in Normal Human Pregnancy

3.1 Introduction

As discussed in detail in *Chapter One* while inhibins were originally identified and isolated from gonadal tissue it was soon apparent that a number of other tissues might produce and secrete inhibins, including the placenta. The presence of inhibin-like bioactivity in the placenta was first demonstrated using aqueous extracts of rabbit placentae to suppress FSH secretion from dispersed rat anterior pituitary cells *in vitro* (Hochberg *et al* 1981). Subsequently, the development of an inhibin radioimmunoassay (McLachlan *et al* 1986b) afforded the observation that a substance with inhibin bioactivity and inhibin immunoactivity that diluted in parallel to human follicular fluid was present in the human placenta (McLachlan *et al* 1986a). Messenger mRNA for the inhibin α-subunit was also identified in a human placental cDNA library (Mayo *et al* 1986) and inhibin-like activity immunolocalised within the placenta (Petraglia *et al* 1987). Studies of placental extracts (Healy *et al* 1988) and primary trophoblast cell cultures (Petraglia *et al* 1987) also confirmed that the placenta yielded large concentrations of inhibin immunoactivity.

Considering these data, that circulating serum levels of immunoreactive inhibin in pregnancy are much higher than observed in non-pregnancy (McLachlan *et al* 1987, Abe *et al* 1990, Kettel *et al* 1991, Tabei *et al* 1991, Tovanabutra *et al* 1993) and that after delivery of the placenta inhibin levels become undetectable within 4 - 5 days (Abe *et al* 1990, Kettel *et al* 1991, Qu and Thomas 1992, Tovanabutra *et al* 1993) the placenta is currently considered the major, if not sole, source of inhibin in human pregnancy (Qu and Thomas 1995).

However, all of the aforementioned studies that reported circulating levels of inhibin were performed using non-specific immunoassays and were unable to discern levels of bioactive, and so presumably physiologically important, inhibin forms from inactive free inhibin subunits. Indeed, while the immunoassays revealed a biphasic pattern of inhibin secretion in pregnancy, with an initial peak of inhibin levels at 9 -10 weeks (Abe *et al* 1990, Tabei *et al* 1992, Tovanabutra *et al* 1993) then a fall to a plateau between 15 and 30 weeks, rising thereafter so that the highest levels are achieved by term (Abe *et al* 1990, Tabei *et al* 1992, Qu and Thomas 1992, Tovanabutra *et al* 1993), bioactive inhibin levels appeared to rise steadily throughout pregnancy without the biphasic profile (Qu and Thomas 1991). This apparent difference between the ontogeny of inhibin bioactivity and immunoactivity would be consistent with a relative excess of inactive forms in early pregnancy, mirroring the relative excess of inhibin α-subunit mRNA at this gestation (Petraglia *et al* 1991). However, more recently,

using an ELISA specific for inhibin-A, it was reported that maternal serum inhibin A levels in pregnancy display a biphasic profile (Muttakrishna *et al* 1995, Illingworth *et al* 1996) consistent with the inhibin immunoreactive data rather than the bioactive data. Furthermore, there has been some debate about the relative contributions of the placenta and the ovary to maternal serum levels of inhibin in very early pregnancy (McLachlan *et al* 1987, Lenton *et al* 1991, Santoro *et al* 1992, Illingworth *et al* 1996, Rombauts *et al* 1996).

This series of studies were undertaken to explore more fully the biology of inhibins in early human pregnancy by describing the levels of inhibins in different pregnancy compartments at different gestations in chromosomally normal singleton and twin pregnancies.

3.2 Materials and Methods

3.2.1 Matched first trimester maternal serum, amniotic and extra-embryonic fluid samples

Matched samples of maternal serum (MS), extra-embryonic coelomic fluid (EECF) and amniotic fluid (AF) from 20 pregnancies were generously supplied by Professor Tim Chard, St Bartholomew's Hospital, London. The EECF and AF had been obtained by transvaginal ultrasound guided needle aspiration prior to the termination of pregnancy under general anaesthesia, as described previously (Wathen *et al,* 1991). All pregnancies were between 8 and 11 weeks of gestation at the time of surgery, calculated by measurement of the crown-rump length (Drumm *et al* 1976), and were confirmed viable by ultrasound. Women with vaginal bleeding were not included in the study. The MS samples were obtained prior to the induction of anaesthesia. This study had local hospital ethical approval.

3.2.2 Maternal serum samples.

Sera were provided through collaboration with Dr David Aitken and Dr Jenny Crossley, both of the Duncan Guthrie Institute of Human Genetics, Glasgow. The sera had been collected prospectively, as part of the routine West of Scotland Down's syndrome and neural tube defects prenatal screening programme and from an ongoing first trimester study of maternal serum markers, and retrieved from storage in Glasgow for this study. Specifically, sera from chromosomally normal singleton and twin pregnancies were identified, through the exclusion of chromosomally abnormal pregnancies, and retrieved from storage. All chromosomally abnormal pregnancies are

identified by the screening programme, either through early pregnancy karyotyping or birth records where karyotyping is not been performed. At the time of collection each sample had been centrifuged and the serum separated within three days of collection and stored at -20°C. For this study, the retrieved sera were transported in dry ice to Edinburgh for assay.

The case-records of a random cohort of the samples were also retrieved affording details of the sex of the babies from the pregnancies studied

3.2.3 Amniotic fluid samples.

Aliquots of amniotic fluid (AF) were obtained through collaboration with Dr Pat Ellis and Mrs Mary Shade, both of the regional cytogenetics laboratory in Edinburgh, Scotland. These had been collected prospectively as part of the clinical amniocentesis service in South East Scotland. In each case AF has separated from fetal epithelial squamous cells by centrifugation at 250 x g either on the day of collection or on the day after. An aliquot of each AF sample was then taken specifically for this study and stored at -20°C prior to assay. The karyotype from each sample was reported subsequently.

3.2.3 Matched maternal serum and amniotic fluid samples.

In 47 women venepuncture was performed immediately prior to amniocentesis. In each case amniocentesis was performed for maternal age and all samples were collected at between 14 and 16 weeks of pregnancy. The blood was centrifuged and the serum separated on the day of collection and stored at -20°C until assay. The AF was processed by the cytogenetics laboratory as detailed above. In each case the karyotype was subsequently reported as normal.

3.2.5 Umbilical cord serum samples.

Umbilical blood, both arterial and venous, was collected from 45 normal singleton pregnancies. In each case, labour onset had been spontaneous and at term (37-41 weeks of pregnancy). After delivery of the baby the cord was double clamped and blood taken after completion of the third stage of labour (delivery of the placenta). The blood, both arterial and venous, was centrifuged on the day of collection, the serum separated and stored at -20°C until assay.

For each of the sample sets (EECF, AF, MS or umbilical cord serum) the gestation at the time of sampling was calculated from certain menstrual dates or from an early pregnancy ultrasound scan and is reported in completed weeks of pregnancy.

3.2.6 Inhibin assays.

In all of the sample sets inhibin-A was measured using the fully modified two-site ELISA as described in *Chapter Two* (page 19). Similarly, inhibin-B and pro-αC containing inhibins were measured using the ELISAs described and validated in *Chapter Two* (page 22 and 24, respectively).

3.2.7 Statistical analyses.

All statistical analyses were performed using Statview 4.1 (Abacus, USA) and SPSS for Windows (SPSS Inc, USA). Where appropriate, regressed medians were calculated to compare the ontogeny of the inhibins in each compartment and multiples of the median (MoM) were used to correct for gestational changes, allowing group comparisons across gestations. The regression equations were kindly calculated by Dr Jenny Crossley (see *Acknowledgments*, page viii).

The regression equations calculated were:

AF inhibin-A: median= $e^{(11.3209 - 84.616/\text{gestation})}$

AF inhibin-B: median= $e^{(9.3254 - 41.656/\text{gestation})}$

MS inhibin-A: median = $-0.0129006x^5 + 0.988003x^4 - 29.6031x^3 + 434.528x^2 - 3136.00x + 9078.01$, (where x = gestation).

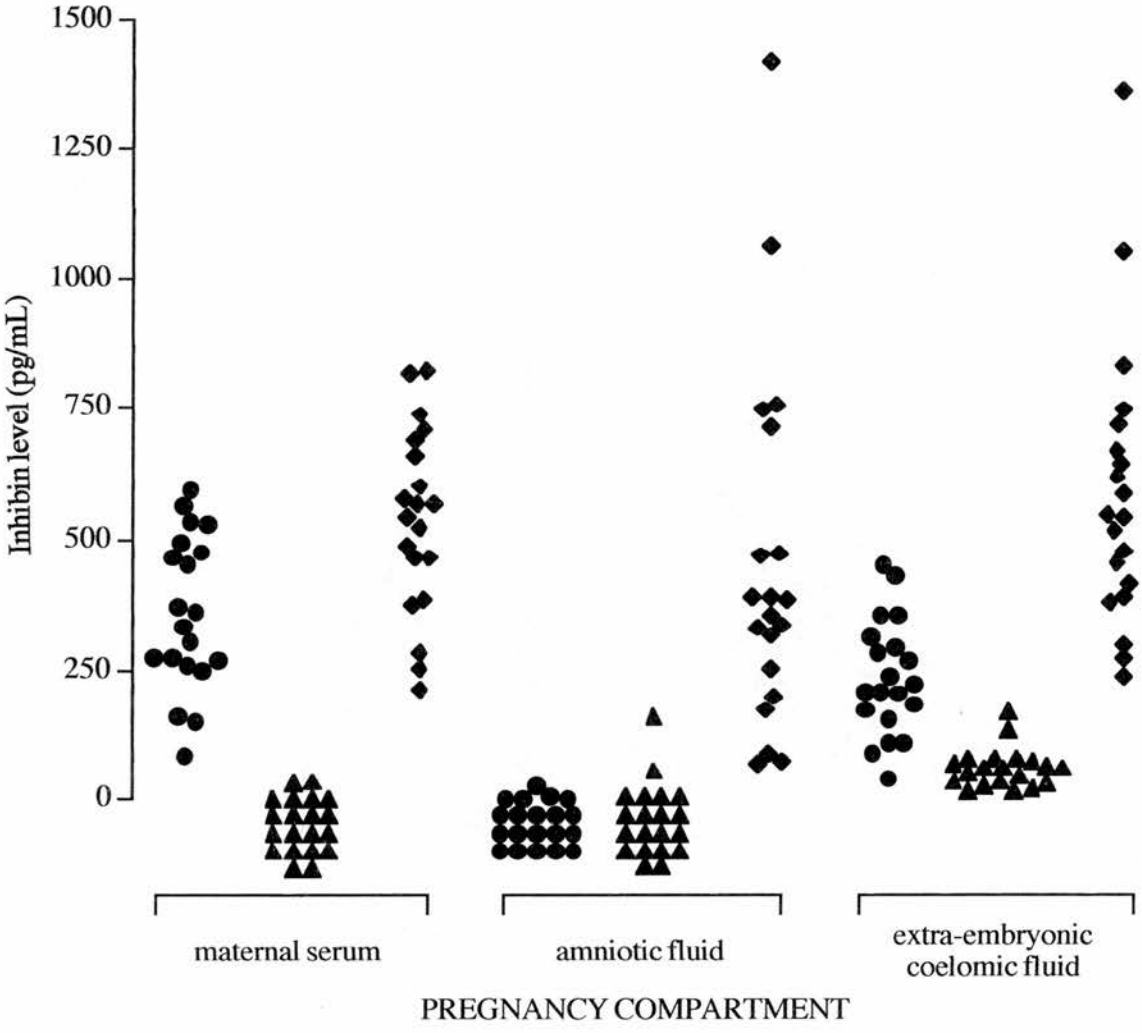
3.3 Results

3.3.1 Inhibins in First Trimester Pregnancy Compartments.

There were insufficient cases (n=20) to be able to discern any changes in protein levels with gestation and therefore the results for all 20 pregnancies have been grouped together and analysed by protein and by pregnancy compartment, with no assessment of the effect of gestation. Figure 3.1 and table 3.1 display the individual sample results and the median (±SEM) concentration of each protein in MS, AF and EECF, respectively.

Figure 3.1

Levels of inhibin-A (●), inhibin-B (▲) and pro-αC containing inhibins (◆) in MS, AF and EECF in 20 pregnancies at 8-11 weeks.



Inhibin-A was present in MS and EECF with concentrations significantly higher in MS ($p=0.04$, Wilcoxon rank sum), but was undetectable in AF, except for one sample. Inhibin-B was present in EECF but was undetectable in both AF and MS. In EECF, there was significantly more inhibin-A than inhibin-B ($p<0.0001$, Wilcoxon rank sum). Pro-αC containing inhibin was detectable in all three compartments, with no significant differences between levels in the compartment (Wilcoxon rank sums; all $p>0.05$). Concentrations of the immunoreactive pro-αC form were significantly higher than the other two proteins in extra-embryonic coelomic and amniotic fluid and in maternal serum ($p< 0.0001$, MS inhibin-A v MS pro-αC; $p<0.0001$ EECF inhibin-A v pro-αC; $p<0.0001$ EECF inhibin-B v pro-αC; all Wilcoxon rank sums).

Table 3.1

Levels of inhibin-A, inhibin-B and pro-aC containing inhibin (pg/mL, median ±SEM) in 20 matched samples of EECF, AF and MS at 8-11 weeks of pregnancy

	Inhibin-A	Inhibin-B	pro-αC
Extra-embryonic coelomic fluid	236.0 ±24.8	62.0 ±8.6	591.7±60.5
amniotic fluid	-	-	452.4±76.8
maternal serum	360.2±32.9	-	539.4±39.5

The levels of immunoreactive pro-αC in all three compartments were not significantly associated with each other nor with levels of inhibin-A in MS. In EECF, and inhibin-A were significantly associated (r=0.62, p=0.003), but not pro-αC inhibins and inhibin-B (r=0.12, p=0.61). Inhibin-A and inhibin-B in EECF were weakly but not significantly associated (r=0.42, p=0.06).

3.3.2 Dimeric inhibins in maternal serum from 10 to 20 weeks of pregnancy.

Inhibin-A and inhibin-B were measured in maternal sera from 807 chromosomally normal singleton pregnancies from 10 to 20 completed weeks of pregnancy. Table 3.2 (page 40) details the distribution of these sera by gestation and the median (10^{th} - 90^{th} percentiles) inhibin-A levels. Figure 3.2 (page 41) displays the regressed data, showing that inhibin-A levels fell from 10 weeks to a nadir at 17 weeks, rising again to 20 weeks.

Sexing information was available from the case-records of 165 (20%) of the 807 pregnancies. 91 (55%) of these 165 pregnancies were males and 74 (45%) were females. These were distributed across the gestational ages as detailed in table 3.3 (page 40). When expressed as multiples of the median (MoM), to correct for gestation, there were no differences between the males and females (1.02 MoM v 1.04 MoM for male and females respectively, p=0.88, Mann-Whitney U test).

Inhibin-A was measured in MS from 268 chromosomally normal twin pregnancies between 10 and 20 weeks of gestation. Table 3.4 shows the 10^{th}, 50^{th} and 90^{th} percentiles for inhibin -A in these pregnancies. No significant differences with gestation were apparent (p>0.05). However, when compared to the MS inhibin-A levels observed in the 807 singleton pregnancies, levels in twin pregnancies were significantly higher (Mann-Whitney U test, p<0.001), being approximately doubled.

Table 3.2

Median, 10th and 90th percentile inhibin-A levels (pg/mL) in maternal serum from 807 chromosomally normal singleton pregnancies

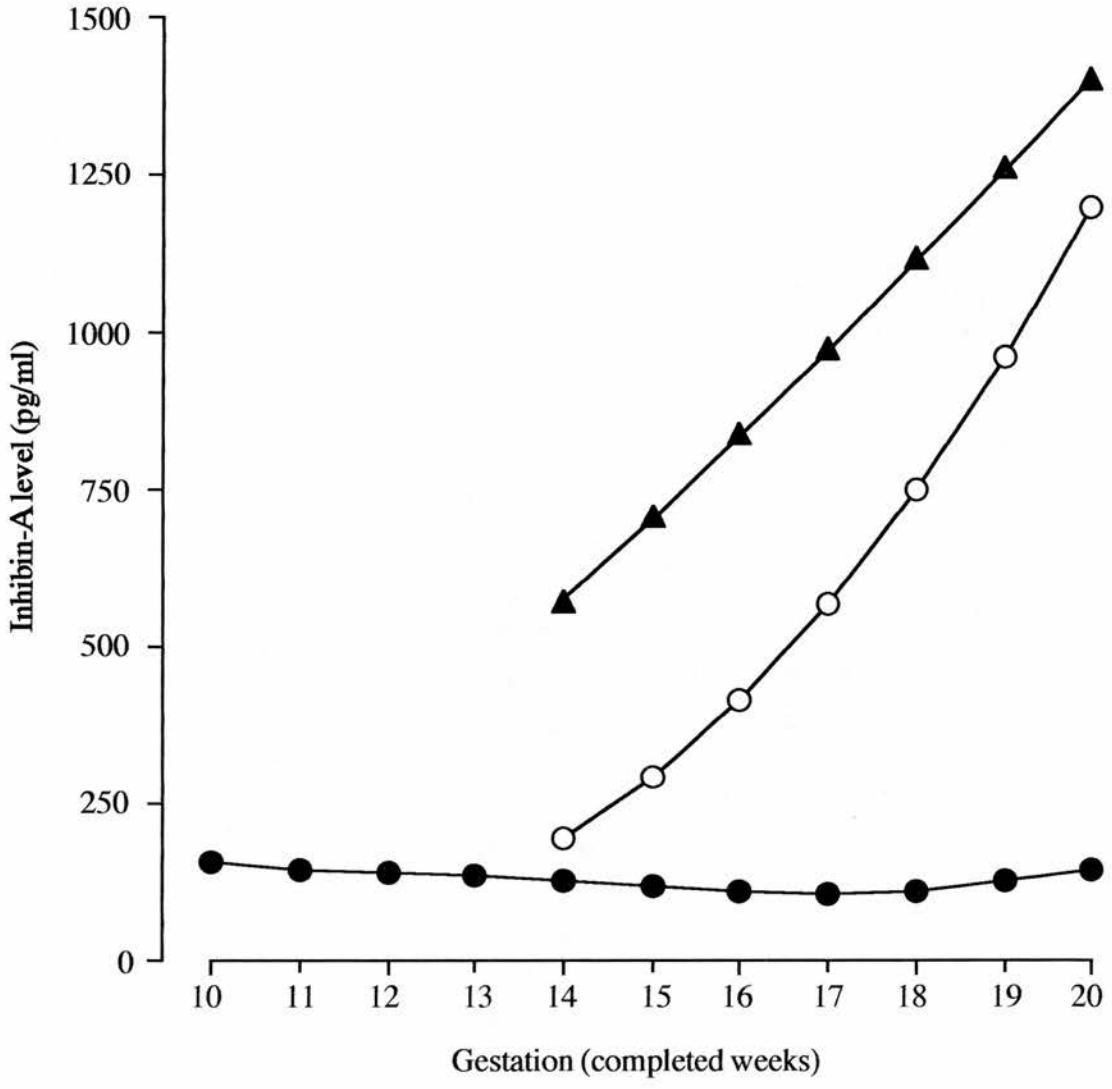
Gestation (completed weeks)	No. of samples	10th	50th	90th
10	75	101.4	177.5	290.7
11	75	88.8	164.3	277.8
12	75	86.8	159.1	253.5
13	75	62.3	133.9	212.2
14	75	73.8	157.4	295.0
15	75	73.2	142.5	261.2
16	75	54.2	119.2	189.4
17	75	59.5	111.9	200.3
18	75	67.3	156.0	291.2
19	75	61.2	146.2	180.3
20	57	74.1	277.4	327.2

Table 3.3

Fetal sex and gestation of 165 chromosomally normal singleton pregnancies

Gestation (weeks)	Male (n)	Female (n)
11	2	0
12	1	2
13	2	0
14	3	6
15	14	23
16	21	12
17	11	12
18	21	10
19	9	7
20	7	2
Total	**91**	**74**

Figure 3.2 Regressed median maternal serum levels of inhibin-A (●) and amniotic fluid levels of inhibin-A (▲) and inhibin-B (O).



3.3.3 Dimeric inhibins in early second trimester amniotic fluid.

Amniotic fluid samples from 603 amniocenteses were available for measurement of inhibin-A content. These represent consecutive chromosomally normal pregnancies. Table 3.5 details the indications for the amniocenteses, showing that 388 (64.3%) were unselected with regard to placental function (group A). The remaining 215 (35.7%) amniocenteses (group B) had been performed following an abnormal result from a screening test.

Table 3.4

Median, 10th and 90th percentile inhibin-A levels (pg/mL) in maternal serum from 268 chromosomally normal twin pregnancies

Gestation (completed weeks)	No. of samples	10th	50th	90th
10	11	192.1	253.0	314.1
11	18	193.3	360.8	630.3
12	9	160.0	286.8	380.5
13	6	46.9	203.6	330.6
14	6	98.5	246.4	391.0
15	57	124.4	231.1	457.0
16	85	140.9	256.9	450.2
17	34	144.6	246.2	532.7
18	10	138.0	270.0	620.8
19	7	122.6	176.8	467.0
20	2	-	351.7	-

Expressed as multiples of the normal singleton regressed median (MoM), the median inhibin-A level in the twin pregnancies was 2.14 MoM (95% CI 1.99 - 2.31).

Inhibin-B was undetectable in MS.

Table 3.5

Indications for amniocentesis

Group	Indication	Number
A: normal indication	maternal age	350
	anxiety	19
	previous abnormal pregnancy	19
	total	*388*
B: abnormal indication	maternal serum screening	200
	abnormal ultrasound scan	15
	total	*215*
Total		**603**

Inhibin-A levels were analysed for each of these two groups separately, by completed week of gestation and by fetal sex within each group. There were no significant differences in inhibin-A levels between the two groups ($p > 0.05$, Mann-Whitney U test) nor between sexes in either group A or B ($p > 0.05$, Mann-Whitney U test). Data from all AF samples were therefore combined, irrespective of indication for sampling or fetal sex, and analysed by gestational age.

Table 3.6 details the median, 10^{th} and 90^{th} percentiles for inhibin-A in AF in these 603 pregnancies. Levels increased steadily and significantly ($p<0.0001$, Mann-Whitney U test) across the gestational window of 14 to 20 weeks, as evidenced by the regressed medians (figure 3.2, page 41).

Table 3.6
Median, 10th and 90th percentile inhibin-A levels (pg/mL) in amniotic fluid from 603 chromosomally normal singleton pregnancies.

Gestation (wks)	14	15	16	17	18	19	20
number	73	117	87	133	137	47	9
10th percentile	158.2	183.7	206.9	212.7	322.8	282.0	489.4
50th percentile	615	680.2	728.7	997.8	1195.9	1130.7	1336.0
90th percentile	1124.6	1438.8	1389.4	2156.0	2717.4	2608.2	2084.1

Inhibin-B assays were performed on 189 of the 603 AF samples. Where possible (weeks 14 - 18 inclusive) all AF samples were from Group A and 15 samples for each sex were selected at random for each gestation. At 19 and 20 weeks however there were insufficient numbers in Group A alone and 7 samples at each of these gestations (1 female and 6 male at 19 weeks and 3 female and 4 male at 20 weeks) were derived from group B. There were no significant differences in inhibin-B levels between the 23 AF samples from Group A and the 7 from Group B at 19 weeks gestation nor between the sexes at any gestation ($p>0.05$, Mann-Whitney U test). The data were therefore grouped by gestation, combining both sexes within each group. Table 3.7 (page 44) details the median, 10th and 90th percentiles for inhibin-B in these 189 pregnancies. Levels increased steadily and significantly across the gestational window as shown in figure 3.2 (page 41).

Table 3.7

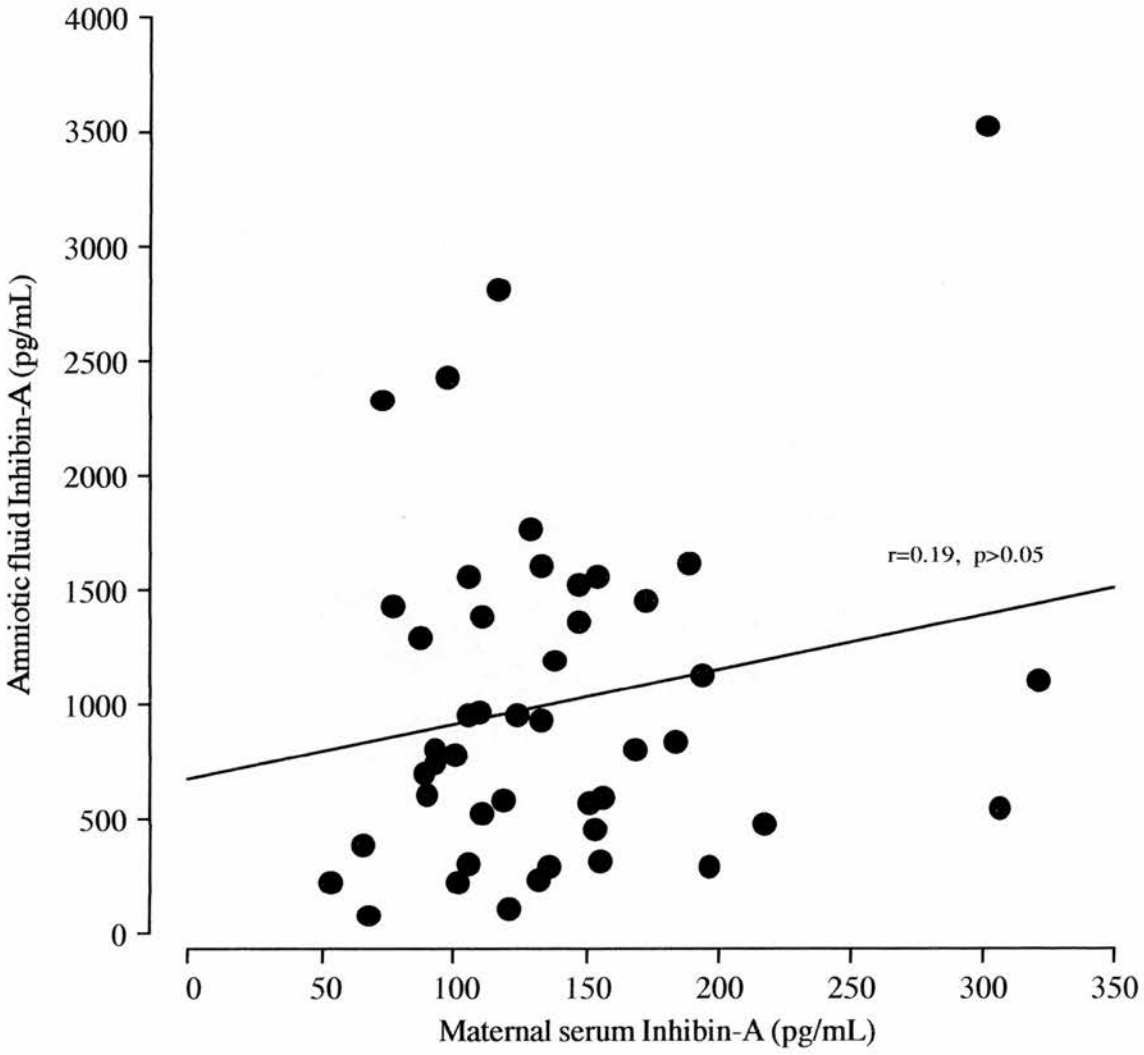
Median, 10th and 90th percentile inhibin-B levels (pg/mL) in amniotic fluid from 189 chromosomally normal singleton pregnancies

Gestation (wks)	14	15	16	17	18	19	20
number	30	30	30	30	30	30	9
10th percentile	67.4	116.0	133.1	114.0	255.6	280.4	439.3
50th percentile	216.6	334.6	261.4	631.8	775.4	1089.2	1078.2
90th percentile	554.6	884.4	1451.8	1738.4	1887.5	2874	2482.5

The rate of increase of inhibin-A and inhibin-B levels in AF differed. At 14 - 16 weeks gestation there was significantly more inhibin-A than inhibin-B ($p<0.001$, Mann-Whitney U test), but this became non-significant at 17 weeks because of the more rapidly rising levels of inhibin-B (figure 3.2, page 41). There was a significant but weak association between the levels of inhibin-A and inhibin-B in the 189 amniotic fluids from which data for both proteins were available (correlation coefficient =0.42, $p=0.0001$)

Inhibin-A was measured in matched maternal sera and AF samples collected from 47 women at 14 to 16 weeks gestation. The median (10th - 90th percentiles) inhibin-A levels in MS and AF were 123.7 (79.0 - 196.1) pg/mL and 802.5 (250.6 - 1731.4) pg/mL, respectively. The AF inhibin-A levels were significantly higher than those in MS (paired t-test, $p<0.001$), but there was no significant correlation of the inhibin-A levels in the AF with those in the matched maternal blood (correlation coefficient = 0.19, $p>0.05$, figure 3.3, page 45)

Figure 3.3
Inhibin-A levels (pg/mL) in 47 matched pairs of maternal serum and amniotic fluid at 14 to 16 weeks of pregnancy.



3.3.4 Dimeric inhibins in fetal serum at term.

Cord blood was examined from 45 consecutive term (37-41 weeks) pregnancies. Of these, 24 (53%) were from a female baby and 21 (47%) from a male, as evident on examination immediately after delivery. Karyotyping was not performed on these babies but clinical examination was normal in each case and all were discharged home with their mother after an uneventful immediate post-natal period.

Inhibin-A was undetectable in the cord serum samples, both arterial and venous, from all 45 pregnancies. Inhibin-B was undetectable in the cord serum from all 24 female babies but was present in serum from all 21 male babies, with no differences between

arterial or venous blood levels. The median (10th - 90th percentile) inhibin-B level in the umbilical vein was 167.4 (111.2 - 224.8) pg/mL. As expected, gestation and birthweight were significantly associated ($r=0.56$, $p<0.01$) but there was no association between cord inhibin-B level with either gestation ($r=0.005$, $p=0.98$) or birthweight ($r=0.11$, $p=0.6$).

3.4 Discussion and conclusions

These are the first data describing the distribution of specific inhibins in first and second trimester pregnancy compartments. In summary, prior to 12 weeks of pregnancy, the approximate timing of the fusion of the amnion and chorion, inhibin-A, inhibin-B and pro-αC containing inhibin are present in EECF, inhibin-A and pro-αC inhibin are present in maternal serum and only pro-αC containing inhibin is present in AF. After 12 weeks however, both inhibin-A and inhibin-B become detectable in AF while inhibin-A remains the only dimer in MS with AF levels of inhibin-A approximately five fold those in MS. In contrast, in serum from the term fetus inhibin-A is undetectable, in both males and females, whereas inhibin-B is present in males. It is proposed that these data, derived from studies using high resolution ultrasound to selectively sample the different early pregnancy compartments, as previously described (Jauniaux *et al* 1991, Wathen *et al* 1991) and illustrated in figures 3.4 and 3.5 (pages 47 and 48), at different gestations, have afforded some clarification of the biology of inhibin in pregnancy.

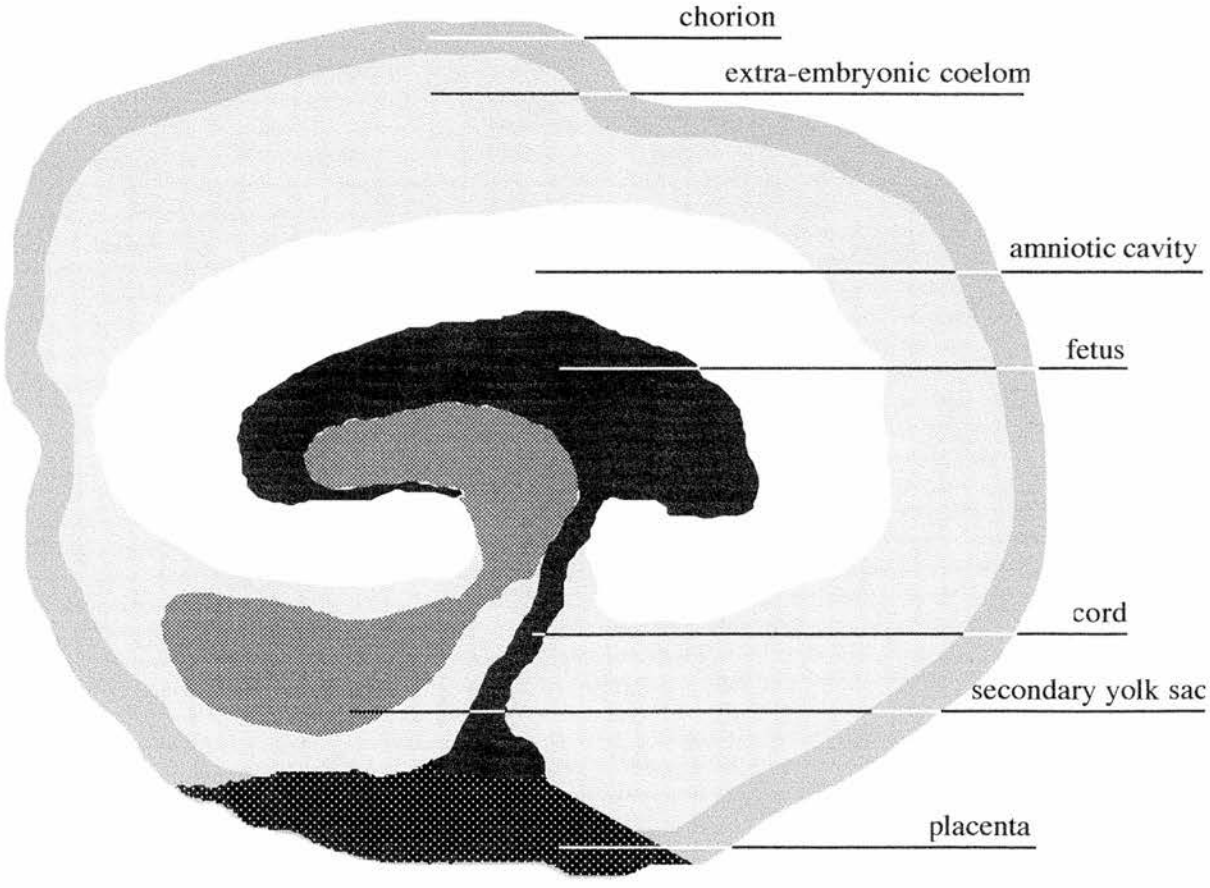
While the current understanding of inhibin secretion in pregnancy is that the placenta is the major source, as reviewed earlier in this chapter and also in *Chapter One*, and by Qu and Thomas (1995), the suggestion that the placenta may not be the single most significant source of hCG (Chard *et al* 1995) and the observation that the ontogenies of hCG and inhibin are similar (Tovanabutra *et al* 1993) raises obvious similar questions over the source(s) of inhibin in pregnancy. Considering the tissue distribution of the mRNAs for the inhibin subunits (Meunier *et al* 1988, Petraglia *et al.* 1990, Tuuri *et al* 1994) there are a number of possible sources of inhibins in early pregnancy including the maternal ovary (specifically the corpus luteum), the decidua, the placenta, the fetal membranes and the fetus. While the corpus luteum is probably the predominant source of inhibin in MS for the first six weeks of pregnancy (Illingworth *et al* 1996), and will increase inhibin-A secretion under pharmacological manipulation (Rombauts *et al* 1996, Illingworth *et al* 1996), MS inhibin-A levels are actually quite low and stable until the luteo-placental shift when levels increase rapidly (Illingworth *et al* 1996).

Thus, in the normal pregnancies studied here, which are all of a gestation beyond the luteo-placental shift, it is likely that the corpus luteum is not a significant source of inhibin-A.

Similarly, although the decidua expresses mRNAs for inhibin-α and both inhibin-ß subunits (Petraglia *et al.* 1990) a number of observations argue against the decidua as a significant source of inhibins. The decidua preferentially expresses β_B subunit mRNA (Petraglia *et al.* 1990), suggesting that it would secrete more inhibin-B than inhibin-A, the converse of what we have observed. Further, other decidual products such as prolactin

Fig 3.4

A schematic representation of a human pregnancy at approximately six weeks gestation showing the different pregnancy compartments.

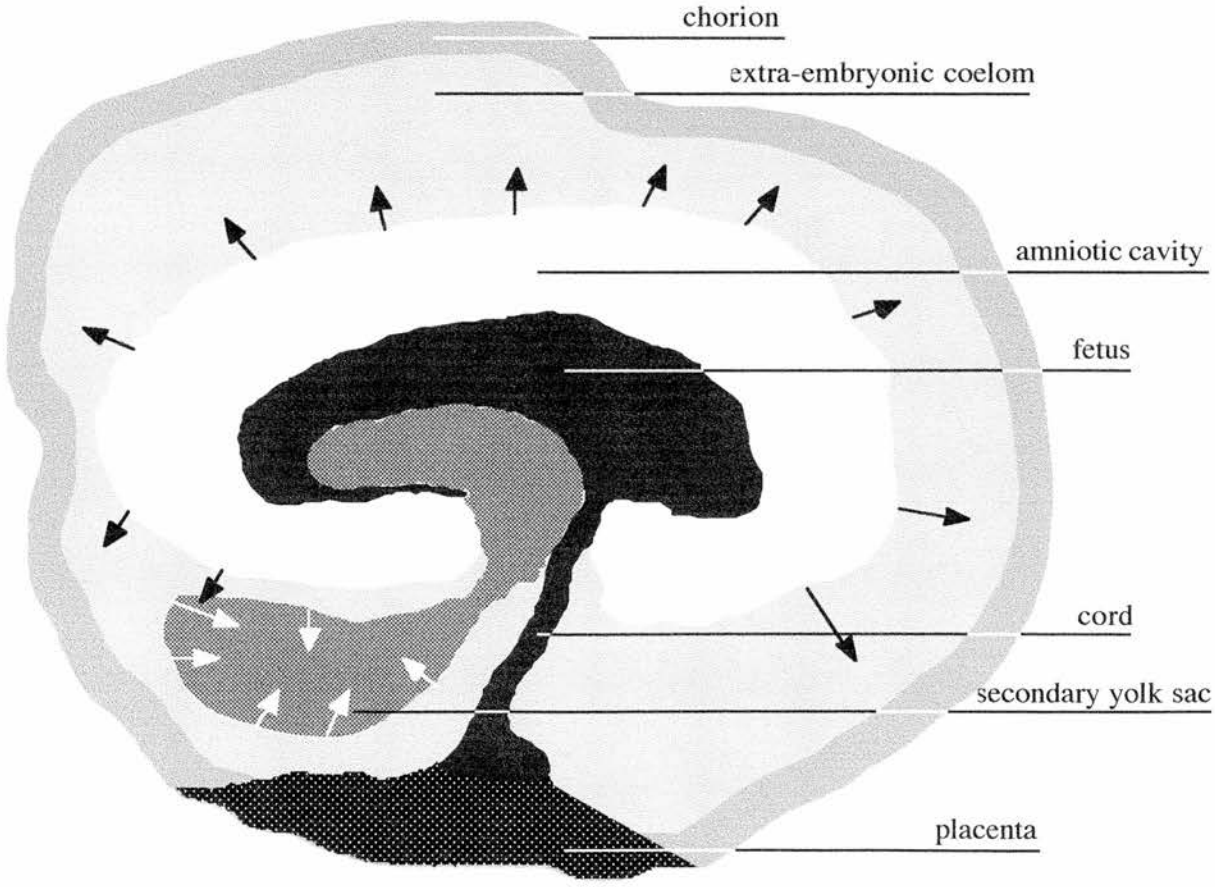


(Golander *et al* 1978) and placental protein 14 (pp14), which is actually an endometrial product (Julkunen *et al* 1985), are found in both the maternal serum and in the intra-uterine cavities (EECF and AF) with the highest levels in the latter (Wathen *et al*

1992, 1993, Kletsky *et al* 1985). In contrast, the levels of inhibin-A are highest in maternal serum. This does not entirely exclude a decidual contribution of inhibin-A to MS and EECF and indeed may simply reflect either a placental, or, less likely, an ovarian contribution to MS inhibin content in early pregnancy (Santoro *et al* 1992, Illingworth *et al* 1996, Rombauts *et al* 1996). However, the significant association between the levels of the two inhibin dimers in EECF suggests a common source for the inhibins in this compartment and since inhibin-B is not detectable at all in maternal serum, indicating that the decidua is not the source of inhibin-B in EECF, the decidua appears a most unlikely source for inhibin-A. The secondary yolk sac, which is enclosed within the extra-embryonic coelom (figures 3.4, page 47, and 3.5) is also an unlikely source of EECF inhibin-A because it degenerates at the end of the first trimester when dimeric inhibin concentrations are increasing.

Figure 3.5

Schematic representation of the progressive changes in the *in utero* pregnancy compartments with increasing gestation. (Arrows represent direction of change).



The fetal membranes and the placenta are therefore the likely sources of inhibins in the compartments studied. Of the two fetal membrane layers, the amnion selectively expresses β_B-subunit mRNA (Petraglia *et al* 1993) which is more indicative of activin production. The chorion however, expresses mRNA for both the β_A- and α-subunits (Petraglia *et al* 1993), consistent with inhibin secretion, and is therefore the likely source of inhibins in EECF and AF. Indeed, the presence of inhibin-A and inhibin-B in EECF, but not AF, at gestations prior to amnion-chorion fusion and the increasing levels of both dimers in AF thereafter is similar in ontogenetic pattern to sex steroids and hCG (Iles *et al* 1992, Atkinson *et al* 1996) which are known products of the chorion. That the chorion may be the principal source of inhibins in these *in utero* compartments would also be in keeping with the extremely high inhibin-A levels observed *in utero* compared to levels in maternal serum in the second trimester, an observation made previously for immunoreactive inhibin (van Lith 1994, Yokhaichiya *et al* 1991). The presence of inhibin-A but total absence of inhibin-B in MS however, suggests that either the chorion selectively secretes inhibin-A into maternal serum or that there are different sources of inhibins for AF and MS. A number of observations would support the latter.

Firstly, the ontogenies of inhibin secretion in the two compartments differ. In MS inhibin-A displays a biphasic profile with levels reasonably stable between 14 and 20 weeks of gestation when AF levels of both inhibin-A and inhibin-B are increasing rapidly. Secondly, in the study of matched MS and AF there was no significant correlation between AF inhibin-A and MS inhibin-A, suggesting separate and differentially regulated sources. The other source is almost certainly the placenta. In the pregnancies studied at 8-11 weeks of gestation the distribution of inhibin-A is identical to that of pregnancy-associated plasma protein A (PAPP-A), a placental product, with higher levels in maternal serum than in extra-embryonic coelom and an absence in amniotic fluid (Iles *et al.*, 1994). Taken together, these data therefore imply that, in early pregnancy, the placenta is the major contributor to MS inhibin levels while the chorion secretes inhibins into AF. The data would also imply that the amnion is not permeable to inhibins until chorion-amnion fusion occurs at approximately 12 weeks. Given the molecular size of inhibins (Robertson *et al* 1995) and that the amnion is impermeable to much smaller molecules (Jauniaux *et al.*, 1991, Gulbis *et al.*, 1992) this would also appear plausible.

Immunoreactive pro-αC inhibin is present in maternal serum in early pregnancy and as for inhibin-A, the corpus luteum is likely the predominant source during early pregnancy (Illingworth *et al.* 1996, Rombauts *et al* 1996). The pro-αC inhibin content

of extra-embryonic coelom is similar to that of maternal serum and may arise from maternal serum or directly from the chorion, as suggested for the two inhibin dimers. The lack of association of immunoreactive pro-αC levels between compartments would be in favour of the latter, as would the association of pro-αC with inhibin-A in extra-embryonic coelom. The presence of pro-αC inhibin in amniotic fluid prior to chorion-amnion fusion is perhaps surprising however. As detailed above, it is unlikely that pro-αC inhibins could traverse the amnion (Jauniaux *et al.*, 1991, Gulbis *et al.*, 1992) suggesting that the amnion itself or the fetus may be the source of these inhibins. While the mRNA for the inhibin α-subunit is expressed in both the amnion (Petraglia *et al* 1993) and in human fetal steroidogenic tissues (Tuuri *et al.*, 1994) it is unclear how the α-inhibins would be secreted by the fetus into the amniotic cavity at this very early gestation, except perhaps by simple leakage. It is therefore suggested that the principal source of immunoreactive pro-αC inhibin in the amniotic fluid prior to 12 weeks is probably the amnion.

Of course, the fetus as a significant source of inhibins cannot be completely excluded. There are certainly similarities between the distributions of inhibin-A and AFP, which is of fetal origin (Wathen *et al* 1991) and immunoreactive inhibin is present in fetal blood (Tabei *et al* 1991, Massa *et al* 1992, Billiar *et al.*, 1995, Rombauts *et al.*, 1996). However, previous studies of immunoreactive inhibin in cord serum concluded that the fetus was not a significant source of inhibin (Abe *et al* 1990, Tabei *et al* 1991, Kettel *et al* 1991), largely because fetal serum levels were lower than maternal levels (Tabei *et al* 1991, Kettel *et al* 1991). While fetal blood at 10-20 weeks of gestation was not available for study, at term there is no inhibin-A in the fetal circulation suggesting that the high levels of inhibin-A in MS, AF and EECF are unlikely to be fetally derived. However, inhibin-B was detectable in cord serum from male, though not female, babies. Indeed, the levels observed are comparable to those reported in adult men (Illingworth *et al* 1996). This finding is in direct contradiction to a previous report that no dimeric inhibin is present in the fetal circulation (Billiar *et al* 1996). That Billiar and colleagues found no inhibin-B can be explained by the capture antibody (E4) used in their "ELISA-B" assay which is an anti inhibin β_A-subunit antibody, displaying no significant affinity for the inhibin β_B-subunit. Interestingly, there are no sex differences in immunoreactive inhibin levels in cord serum at term (Abe *et al* 1990, Kettel *et al* 1991, Khalil *et al* 1995), suggesting that the majority of the inhibin detected in cord serum by the inhibin α-subunit assays used in previous studies (Abe *et al* 1990, Kettel *et al* 1991, Tabei *et al* 1991) was free α-subunit (Billiar *et al* 1995) rather than dimeric inhibin. Importantly, given that no differences in AF inhibin-B levels at

14-20 weeks of gestation were observed between males and females it would appear unlikely that the inhibin-B detected in AF was derived from the fetus. Rather, it remains likely that the chorion is the sole, if not major source, although, as for inhibin-A, no fetal sera at this gestation were available for study.

In keeping with inhibin-B in male but not female sera, the fetal testis expresses mRNA for the inhibin subunits, while the ovary does not (Tuuri *et al* 1994) suggesting that the inhibin-B may be of testicular origin, rather than from another endocrine organ common to both sexes such as the adrenal. If confirmed, these data present the first evidence that the human fetal testis actively secretes dimeric inhibin at term and suggests that the inhibin-B ELISA will afford opportunities for new insights into the control and development of the male fetal hypothalamo-pituitary-gonadal axis. Indeed, at 26-28 weeks immunoreactive inhibin levels are higher in males than females (Massa *et al* 1992), whereas there are no differences at term (Abe *et al* 1990, Kettel *et al* 1991, Khalil *et al* 1995). In light of the inhibin-B data, the immunoreactive inhibin findings at earlier gestations may represent relative changes in inhibin-B, particularly when at these gestations FSH levels in males are significantly lower than in females (Reyes *et al* 1974, Winter 1982, Massa *et al* 1992). This sex difference in circulating FSH has been previously explained by the higher circulating levels of testosterone in males (Reyes *et al* 1974, Winter 1982) but it is possible that it may, at least in part, be due to inhibin-B, being evidence of negative feedback by inhibin-B on fetal pituitary FSH secretion at these gestations. It would certainly now be intriguing to re-visit previous studies of the pituitary-gonadal axis in term and preterm neonates, exploring the possible role(s) of inhibins in the functional development of the axis. In particular, it would be interesting to investigate whether the enhanced gonadotrophin secretion observed in preterm female neonates compared to term neonates (Tapanainen *et al* 1981) corresponds to a transient role for inhibins in the female axis, a role not evident in the simple term observations reported here.

In addition to exploring the source(s) of inhibins in pregnancy the studies reported here have also clarified other areas of inhibin biology in pregnancy. Levels of immunoreactive inhibin in maternal serum throughout pregnancy have been extensively reported previously using inhibin α-subunit based assays (Abe *et al* 1990, Tabei *et al* 1991, Tovanabutra *et al* 1993, Kettel *et al* 1991, Qu and Thomas 1992). Levels rise from ovulation to a peak at 9-10 weeks gestation, falling to a plateau at approximately 15 weeks and thereafter rising in the third trimester to a new peak at term. In contrast, Qu and colleagues (1991) showed that bioactive levels of inhibin, as determined by the suppression of FSH release from a dispersed sheep anterior

pituitary cell assay (Tsonis *et al* 1986), increased steadily, without this biphasic profile, suggesting that the biphasic immunoreactive profile reflected a preponderance of non-bioactive inhibin forms, presumably monomeric inhibin α-subunits, in the early first trimester with increasing circulating levels of dimeric inhibin as pregnancy progresses. This would certainly be feasible given the ability of the inhibin immunoassays to detect free inhibin α-subunit (Schneyer *et al* 1990, also see *Chapter Two*) Furthermore, this interpretation would be in keeping with immunohistochemistry and *in situ* hybridisation studies that have suggested a relative excess of inhibin α-subunit in early pregnancy trophoblast with increasing production of the inhibin β_A- and β_B-subunits as gestation increases. In those studies, there was "intense" expression of the inhibin α-subunit mRNA, less of the β_A-subunit mRNA and no β_B-subunit in first and second trimester trophoblast whereas in late pregnancy trophoblast low levels of β_B-subunit mRNA were detectable for the first time with maximum levels of mRNAs for the other two subunits (Petraglia *et al* 1991). Mirroring the changes in mRNAs, immunostaining for the three inhibin subunits in the placenta reveals a relative excess of α-subunit in early pregnancy with some inhibin β_A-subunit and very weak staining for the β_B-subunit. With increasing gestation, the relative staining for inhibin α-subunit declines as β_A-subunit and β_B-subunit staining increases (Petraglia *et al* 1991, Minami *et al* 1992).

More recently however, studies using inhibin-specific ELISAs (Muttukrishna *et al* 1995, Illingworth *et al* 1996, Rombauts *et al* 1996) have afforded useful new insights into the biology of inhibins in pregnancy. These studies have suggested that of the two inhibin dimers, only inhibin-A is detectable in maternal serum. Indeed, using gel permeation chromatography of pooled pregnancy sera Muttukrishna and her colleagues (1995) demonstrated that of the various molecular weight forms of inhibin that are known to exist in follicular fluid and non-pregnant female serum (Robertson *et al* 1995) only the fully processed 31kDa inhibin-A is present in pregnancy, although data related later in this thesis dispute this latter suggestion (*Chapter Seven*) as do the recent data of Khalil and colleagues (1995). Furthermore, the recent reports have shown that the ontogeny of inhibin-A in pregnancy is biphasic, similar to that of immunoreactive inhibin (Muttukrishna *et al* 1995, Illingworth *et al* 1996) but different from the bioactive data (Qu *et al* 1991). On this much larger series of maternal serum samples than previously reported, and with the normal range series reported in *Chapter Five*, page 72, it is confirmed that the ontogeny of inhibin-A in maternal serum shows a biphasic pattern. However, the data presented here suggests that the secondary rise commences at 18 weeks of gestation, much earlier than previously reported

(Muttukrishna *et al* 1995). These data would therefore suggest that the bioactive inhibin levels previously described may have been confounded by either other FSH-regulating peptides, such as activin which is produced by the placenta and is present in MS (Petraglia *et al* 1995, Yokoyama *et al* 1995, Harada *et al* 1996,Muttukrishna *et al* 19996), or by inadequate stripping of the very high levels of circulating sex steroids that exist in pregnancy (Qu *et al* 1991). The presence of inhibin binding proteins such as follistatin (Robertson *et al* 1987, Ueno *et al* 1987, Shimonaka *et al* 199, Krummen *et al* 1993) and α_2-macroglobulin (Vaughan and Vale 1993) in serum may also confound *in vitro* bioassays. This is a particularly plausible explanation since the levels of binding proteins increase in pregnancy (Studd *et al* 1970) and chromatography has confirmed that active and significant binding of inhibin in pregnancy sera appears to occur (Khalil *et al* 1995).

The mechanisms underlying the biphasic profile of inhibin-A secretion in pregnancy is unknown. However, this secretory pattern is similar to that of hCG (Tovanabutra *et al* 1993) and it has been suggested that the hCG peak at 9-10 weeks of pregnancy may be related to altered chorionic function, secondary to the fusion with the amnion that occurs at this gestation (Chard *et al* 1995). This hypothesis requires the chorionic trophoblast, rather than simply the placental trophoblast, to be a major contributor to hCG in the maternal circulation and this prerequisite would similarly apply to inhibins. Unfortunately, the studies reported here do not explore the possibility that inhibin secretion by the chorion into maternal serum is altered by amnion-chorion fusion although they do suggest that the chorion is a significant source of inhibins, as discussed above.

The higher maternal serum levels of inhibin-A in twin pregnancies were not unexpected. It has been shown previously that immunoreactive inhibin levels are higher in multiple pregnancies (Norman *et al* 1993) than in singletons and that maternal serum levels of hCG in twin pregnancies are approximately double those of singletons pregnancies (Wald *et al* 1991). The median MoM of 2.14 for the twin pregnancies is entirely in keeping with these previous data, suggesting that in twin pregnancies the secretion of inhibins and hCG are increased two-fold over singletons. Whether this is simply related to the increased mass of tissue, either placental, chorionic or both, which seems likely or whether there is a more complex explanation remains unexplored. Nonetheless, the inhibin-A findings reported here have been recently confirmed by another study of 200 twin and 600 singleton pregnancies (Watt *et al* 1996). These authors reported a median MoM of 1.99 for the twin pregnancies, not significantly different from the data reported here.

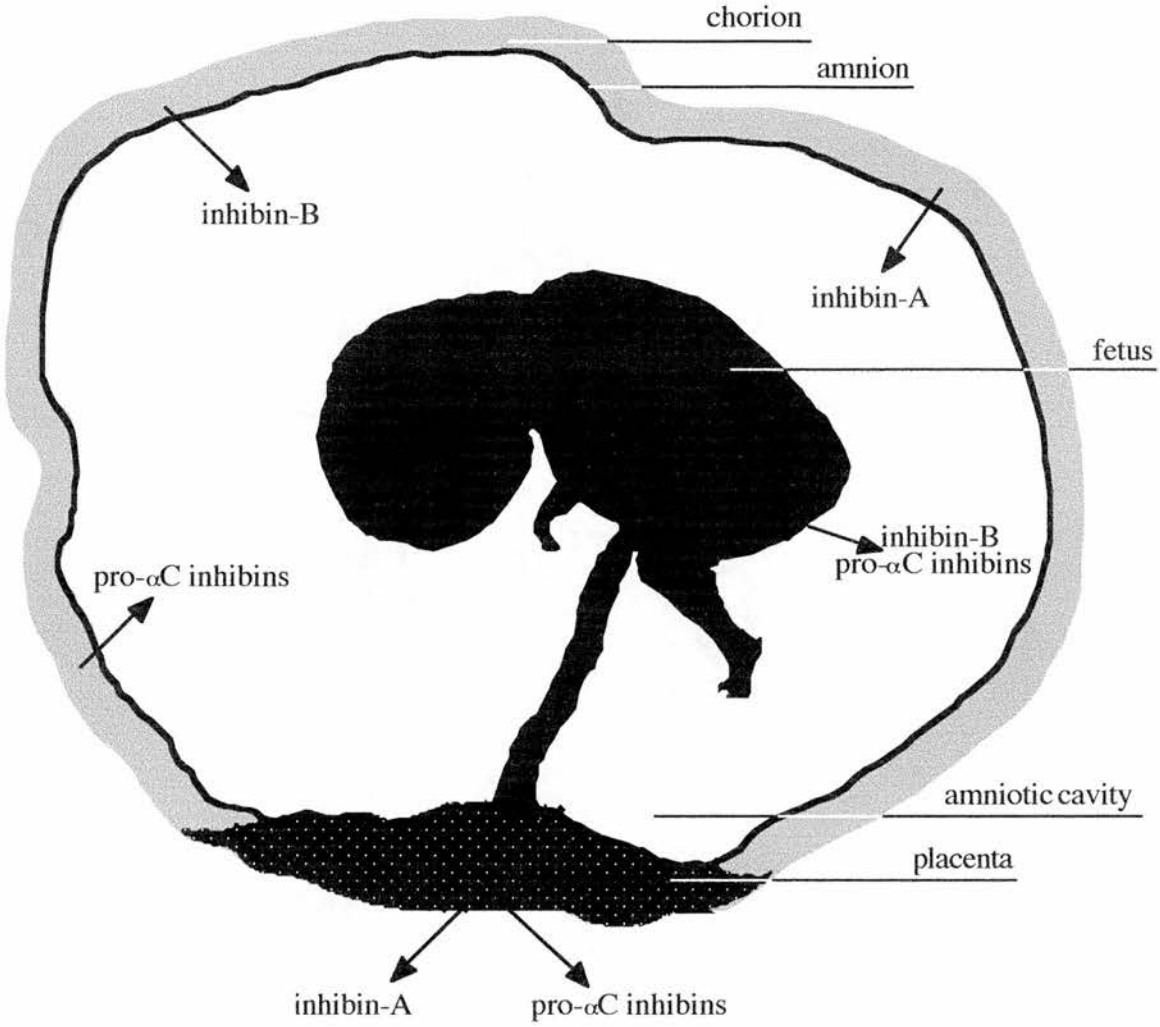
It has been reported previously that the maternal serum immunoreactive inhibin level is sex dependant with higher levels observed in pregnancies with a female fetus (Khalil *et al* 1995), similar to hCG (Obiekwe and Chard 1982, Leporrier *et al* 1992). Such gender differences for inhibin-A were not observed here. Furthermore, no sex-related differences in AF levels of either inhibin-A or inhibin-B at 14 to 20 weeks gestation were observed. Together these data would suggest that either there are gender differences for inhibins not detected by the dimeric inhibin assays, perhaps free inhibin α-subunit, or that the differences are only apparent at later gestations, as is indeed the case for hCG (Muller *et al* 1993), or that the differences observed, using stepwise multiple regression, may have been secondary to the small number of samples studied (n=33) (Khalil *et al* 1995). A study of inhibin-A and pro-αC containing inhibin levels in maternal serum throughout pregnancy in a larger dataset would be useful in exploring these possibilities further and presently it remains unclear whether gender differences in specific inhibin levels exist in later pregnancy or not. However, given that levels of immunoreactive inhibin in fetal serum are lower in females compared to males (Massa *et al* 1992) opposite findings in MS would be most intriguing.

The studies reported here also demonstrate for the first time that inhibin-B is not detectable in MS at 10-20 weeks gestation, extending a previous report that inhibin-B was absent in MS up to 11 weeks gestation (Illingworth *et al* 1996). Interestingly, recent and unpublished data would suggest that inhibin-B is present in MS at term (Petraglia, personal communication) which remains in keeping with the existing molecular and immunostaining data, as previously described, and merits further exploration.

In summary, the data presented in this chapter offer some clarification of the biology of inhibins in pregnancy and are illustrated by figure 3.6, page 55. Further, the data provide evidence to challenge the concept that the placenta is the only significant source of inhibins in pregnancy. It is suggested that the placenta secretes inhibin-A primarily into the maternal circulation while the fetal membranes are the likely major source of both inhibin-A and inhibin-B in EECF and AF. The data also suggest for the first time that the fetal testis secretes inhibin-B, implicating this hormone in the development and control of fetal hypothalamo-pituitary-testicular axis.

Figure 3.6

A schematic representation of a human pregnancy at approximately 12-13 weeks of gestation, illustrating the source and route of secretion of different inhibin forms.



Chapter Four

Immunoreactive Inhibins in Down's Syndrome Pregnancy

4.1 Introduction

As discussed in *Chapter One*, prenatal screening for Down's syndrome has become an important and established part of modern obstetric care. While there are a number of approaches to screening (Cuckle 1994, Brizot *et al* 1994, Brambati *et al* 1994, Borrell *et al* 1996), currently most programmes depend upon maternal age in combination with the measurement of human chorionic gonadotrophin (hCG) and alpha feto-protein (AFP), with or without unconjugated oestriol (uE_3), in maternal serum from 15 weeks gestation. Extensive prospective study, in many countries, has shown that such an approach will detect approximately 65-70% of Down's syndrome pregnancies for an amniocentesis rate of 5% (RCOG 1993). Of course, assessment of new and different serum markers is ever continuing in the hope of increasing detection rates further.

Recently, it was reported that, as might have been expected from the *in vitro* relationship between hCG and inhibin, that second trimester maternal serum levels of immunoreactive inhibin are increased in Down's syndrome pregnancies and that inhibin may be useful as a serum marker for Down's syndrome (van Lith *et al* 1992). On average, the median (95% CI) inhibin level in Down's syndrome pregnancies was 1.9 (1.3-2.8) MoMs, very similar to hCG, though lower than free β-hCG (Macri *et al* 1994). Subsequently, Spencer and his colleagues (1993) reported an even higher median MoM of 3.65, confirming that immunoreactive inhibin levels are indeed elevated in Down's syndrome pregnancies in the second trimester. However, the latter authors suggested that inhibin was unlikely to be a useful prenatal marker because there was a strong correlation with free β-hCG ($r=0.74$), suggesting that inhibin would add little to the performance of screening combinations using free β-hCG, and because the width of distribution of inhibin levels was too broad, implying that there would be unacceptable overlap between normal and affected pregnancies. The only other report of immunoreactive inhibin as a second trimester prenatal marker of Down's syndrome was equally gloomy about the potential value of this protein in a screening context (Cuckle *et al* 1994). Again, the significant association with hCG was cited as the main reason and in addition, in this series the degree of increase of inhibin in the Down's syndrome sera was much less (1.3 MoM, 95% CI 0.9-1.9).

Nonetheless, it remained conceivable that inhibin may be a useful first trimester marker, even if it was not useful in the second trimester. Pregnancy associated plasma protein-A (PAPP-A) is an extremely useful marker of Down's syndrome in the first trimester of pregnancy, with levels of approximately only 0.3 MoM (Brambati *et al*

1991, Brambati *et al* 1993, Muller *et al* 1993, Hurley *et al* 1993) and yet is unable to differentiate between cases and controls in the second trimester (Cuckle *et al* 1992). Therefore, it was decided to assess first trimester maternal serum inhibin levels in chromosomally normal and Down's syndrome pregnancies.

4.2 Materials and Methods

4.2.1 Serum samples

In Lothian, serum is routinely, prospectively collected from all women at the first hospital antenatal visit and stored at -20°C. Eleven women (mean age 31.6 ± 5.1 years) were identified from records of known Down's affected pregnancies (detected by maternal serum AFP at 16 weeks and subsequent amniocentesis), allowing their stored serum to be retrieved. The serum from five of these women had been collected at 11 completed weeks of gestation and the other six at 12 completed weeks, calculated from an ultrasound scan performed on the day of sampling. For each Down's syndrome case four women, matched for gestation (ultrasound determined) and duration of storage, who had had a chromosomally normal pregnancy were identified (mean age 27.5 ± 4.9 years) and their samples retrieved for analysis.

4.2.2 Inhibin assays

Inhibin was assayed using two different immunoassays, as described in detail in *Chapter Two*. The first of the assays (McLachlan *et al* 1986b, 1987) is now distributed by the National Institute of Child Health and Human Development (NICHD) while the second is commercially produced and distributed by Medgenix (High Wycombe, England). Importantly, both of these assays utilise antibodies directed against epitopes on the inhibin α-subunit and are thus able to detect both forms of dimeric inhibin, inhibin-A and inhibin-B, and monomeric α-subunit (Schneyer *et al* 1990). Immunoreactive inhibin detected with these assays do not therefore necessarily reflect bioactive protein.

4.2.3 Statistical analyses

The results obtained from neither assay were distributed normally and data were transformed, using square root and logarithmic transformation for the NICHD and Medgenix assays results, respectively to allow statistical comparisons. Analysis of variance (ANOVA) was performed on the transformed data to assess the effects of gestation and Down's syndrome. Where significance was detected ($p<0.05$), a post-hoc analysis was performed using Duncan's test.

4.3 Results

4.3.1 NICHD Assay

The mean (± SEM) maternal serum immunoreactive inhibin at 11 and 12 weeks gestation in the Down's syndrome samples were 3186 ± 195pg/ml and 2517 ± 441pg/ml and in the controls 2020 ± 172pg/ml and 2561 ± 198pg/ml, respectively. The mean immunoreactive inhibin levels at the two gestations were not significantly different for either group. Figure 4.1 shows the data from both gestations combined, revealing no significant difference between Down's syndrome and normal pregnancies (2821 ± 267pg/ml v 2317± 138pg/ml respectively, n.s.). However, analysing each gestation separately, at 11 weeks the mean serum immunoreactive inhibin in Down's syndrome and control pregnancies was 3186 ± 195 pg/ml and 2020 ± 172 pg/ml, respectively (figure 4.2, page 60). The mean inhibin level at 11 weeks gestation was significantly higher in the Down's affected pregnancies when compared to the controls (Mann-Whitney U test, $p<0.01$). No significant differences existed at 12 weeks (2517 ±441 pg/ml v 2561 ±198 pg/ml) (figure 4.2).

Figure 4.1.
Maternal serum immunoreactive inhibin levels detected by two different immunoassays in 11 Down's syndrome and 44 chromosomally normal pregnancies.

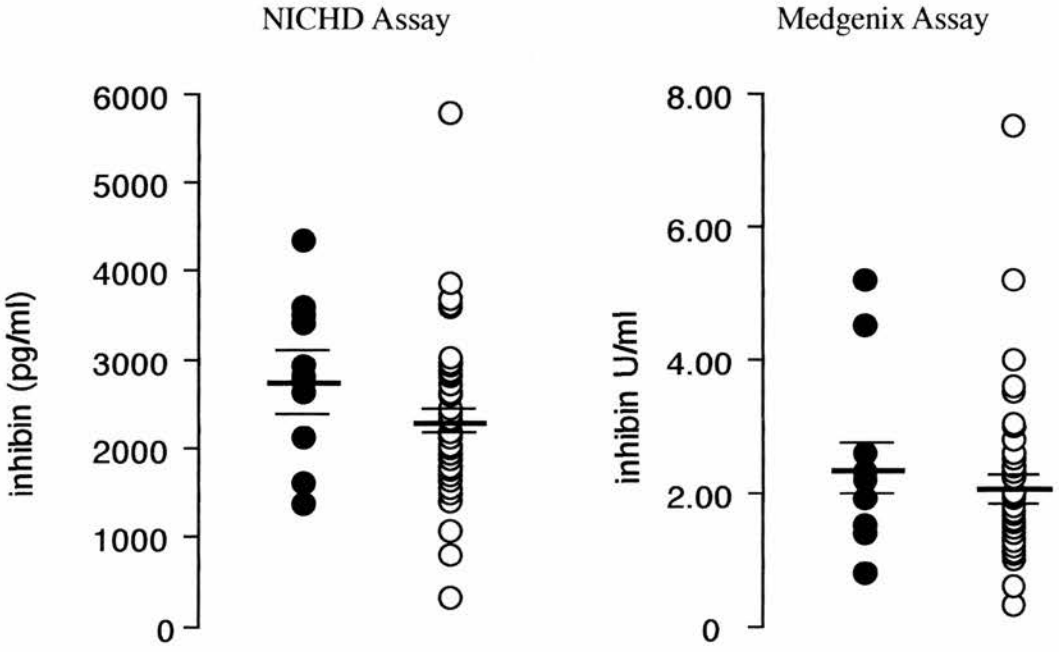
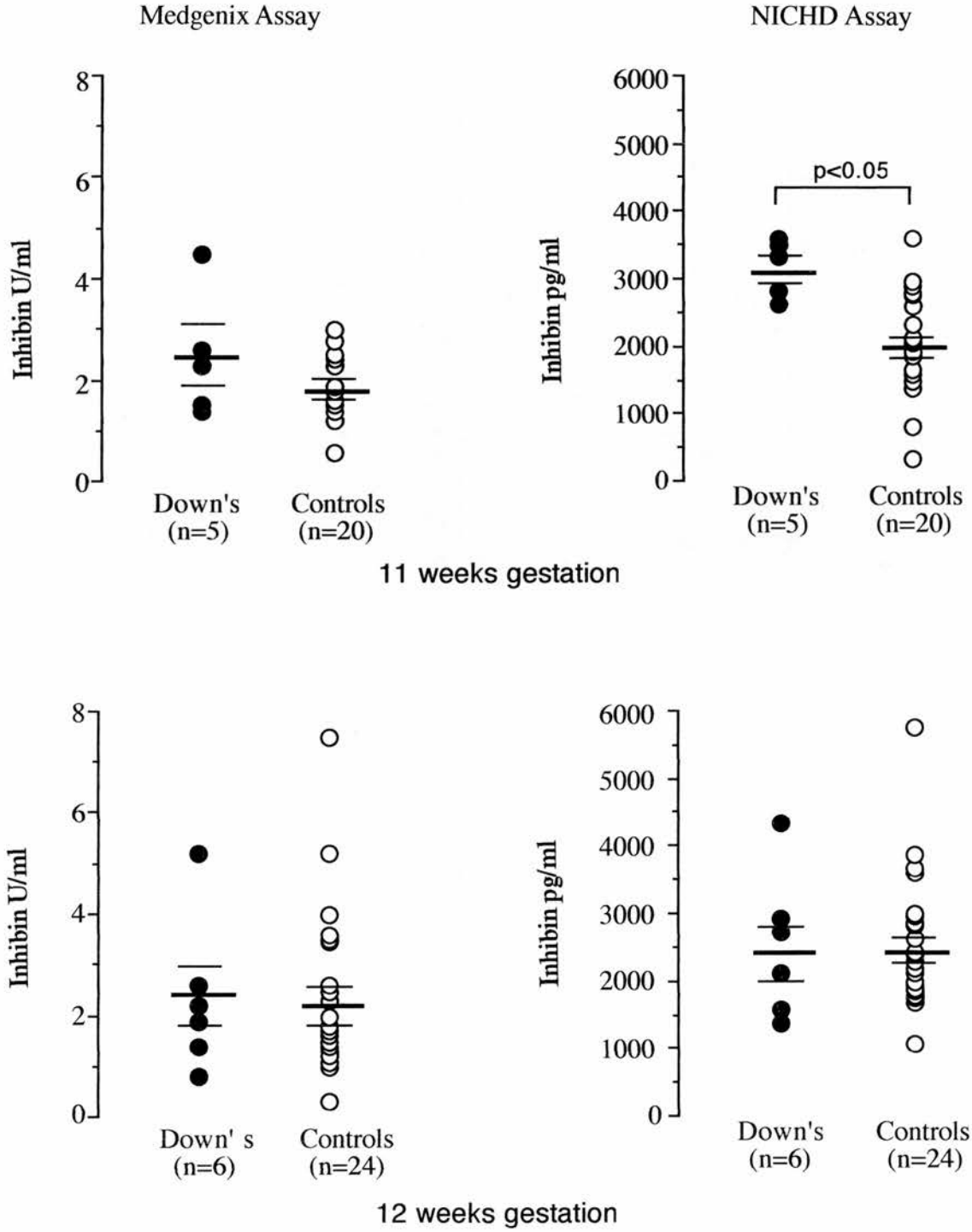


Figure 4.2.

Maternal serum immunoreactive inhibin levels in sera from 11 Down's syndrome pregnancies and 44 control pregnancies at 11 and 12 weeks of pregnancy.



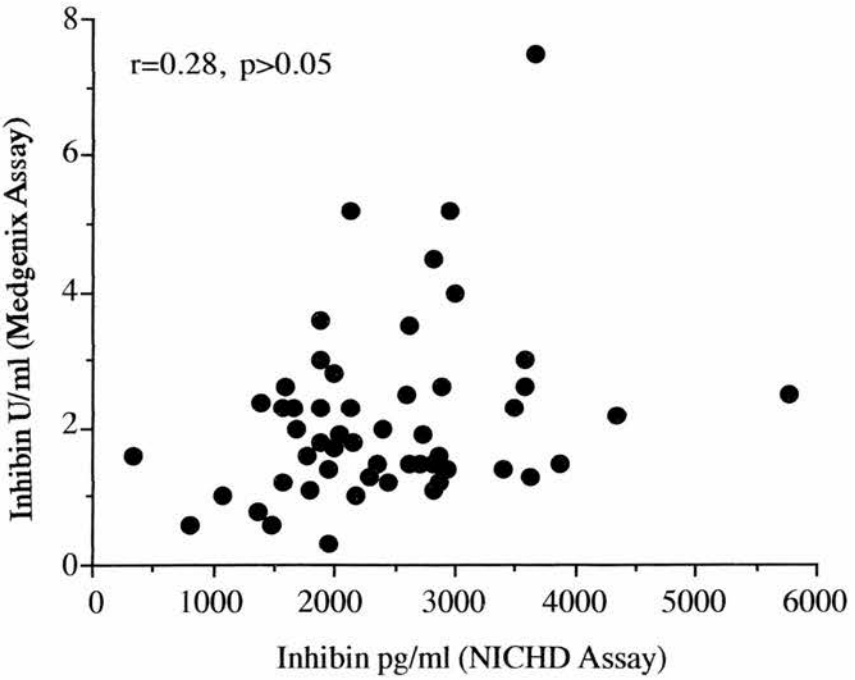
4.3.2 Medgenix Assay

The mean (± SEM) maternal serum immunoreactive inhibin at 11 and 12 weeks gestation in the Down's syndrome samples was 2.46 ±0.56 U/ml and 2.35 ±0.62 U/ml and in the controls 1.84 ±0.17 U/ml and 2.20 ±0.33 U/ml, respectively. The mean immunoreactive inhibin levels at the two gestations were not significantly different for either group. Figure 4.1 (page 59) shows the data from each gestation combined, revealing no significant difference between Down's syndrome and normal pregnancies (2.4 ±0.4 U/ml v 2.04 ± 0.19 U/ml respectively, n.s.). Analysis of each gestation separately also revealed no significant effect on inhibin levels exerted by Down's syndrome (data not shown).

4.3.3 Assay comparisons

There was no significant correlation between the inhibin levels derived from the two assays, r=0.28, p>0.05 (figure 4.3).

Figure 4.3.
Regression plot of maternal serum inhibin levels derived from 2 different immunoassays.



4.4 Discussion and conclusions

Established maternal serum screening programmes for Down's syndrome are currently timed for 15 to 20 weeks of pregnancy (RCOG 1993), utilising maternal age in combination with the measurement of maternal serum hCG, either intact or free β-hCG, and AFP, with or without unconjugated oestriol (uE_3). Recently, it was reported that immunoreactive inhibin may be a useful second trimester marker for Down's syndrome (van Lith *et al* 1992), although this was not a universal interpretation of the elevated inhibin levels observed (Spencer *et al* 1993, Cuckle *et al* 1994). However, even the data of Spencer and his colleagues (1993), who did not believe that inhibin would find a useful role as a prenatal marker of Down's syndrome, suggest that inhibin alone, without maternal age or other serum markers, might offer a detection rate of 66% for a false positive rate of 5.3% - an impressive rate indeed. Nonetheless, in the time since those reports immunoreactive inhibin has not found any further application as a prenatal marker, probably due in part to concerns regarding reproducibility (Cuckle *et al* 1994), unsuitable distribution widths (Spencer *et al* 1993, Cuckle *et al* 1994) and significant associations between inhibin and hCG (Spencer *et al* 1993, Cuckle *et al* 1994).

As reviewed by Macintosh and Chard (1993), a number of fetal and placental products have also been investigated as putative first trimester markers. This study was the first to assess immunoreactive inhibin as a marker for Down's syndrome at this earlier gestation and remains the only study to have examined inhibin levels in Down's syndrome pregnancies, at any gestation, using two different immunoassays. The resultant data show that there is no apparent effect of gestation on maternal serum immunoreactive inhibin, as detected by either assay, at 11 and 12 weeks of pregnancy and that when the data for the two gestations are combined, there is no significant difference between inhibin levels observed in Down's syndrome cases and those in chromosomally normal controls. Again, this finding applied equally to both assays studied. However, using the "NICHD assay", but not the Medgenix assay, a significant difference in maternal serum inhibin levels between Down's syndrome cases and normal controls was apparent at eleven weeks of gestation but not at twelve weeks. The extremely small number of cases in this study necessitates cautious interpretation but the data would not support immunoreactive inhibin, as detected by these two assays, as a useful marker of Down's syndrome at 11 and 12 weeks of pregnancy. A subsequent report from the Dutch Working Party on Prenatal Diagnosis (van Lith *et al* 1994) confirms this conclusion, using only the Medgenix assay. The

isolated significant elevation in serum immunoreactive inhibin at 11 weeks, and not at 12 weeks, is surprising when the previously reports of screening in the second trimester are considered (van Lith et al 1992, Spencer et al 1993, Cuckle *et al* 1994). However, the published second trimester data were obtained using the Medgenix assay and it is possible that the NICHD assay is detecting inhibin species, not detected by the Medgenix antibodies, that are elevated in Down's syndrome in the first trimester. Nonetheless, this isolated finding at 11 weeks gestation suggests that inhibin is unlikely to be a useful marker. In summary therefore, it would appear that immunoreactive inhibin is not a useful marker of Down's syndrome in either the first or second trimester. Table 4.1 displays the median levels of immunoreactive inhibin in the published series to date.

Table 4.1
Maternal serum immunoreactive inhibin levels in Down's syndrome in 5 studies.

Author	Assay	Trimester	Median (95% CI) MoM
van Lith *et al* 1992	Medgenix	Second	1.9 (1.3 - 2.8)
Spencer *et al* 1993	Medgenix	Second	3.65 (1.8- 7.5)
Cuckle *et al* 1994	Medgenix	Second	1.3 (0.9 - 1.9)
Wallace *et al* 1994	NICHD	First	1.3 (1.0 - 1.6)
Wallace *et al* 1994	Medgenix	First	0.88 (0.7 - 1.5)
van Lith *et al* 1994	Medgenix	First	1.3 (0.8 - 2.1)

Perhaps more intriguing was the observation that the inhibin levels obtained with the two assays were poorly correlated, suggesting that different inhibin species were being detected. This may have significant implications for the future of inhibin as a marker and in this context a comparison with hCG as a marker of Down's syndrome in the first and second trimesters is worthwhile.

Although it is nearly ten years since it was first recognised that hCG levels are increased in association with trisomy 21 (Bogart *et al* 1987), and that these levels reflect increased placental mRNA expression for both hCG subunits (Eldar-Geva *et al* 1995), it remains unclear what the mechanisms underlying these changes are. However, it is clear that while maternal serum levels of both intact hCG (Bogart *et al*

1987) and free β-hCG (Macri *et al* 1990) are elevated in Down's syndrome in the second trimester, intact hCG is not elevated in the first trimester (Cuckle *et al* 1988, Aitken *et al* 1993) and yet free β-hCG is (Spencer *et al* 1992, Aitken *et al* 1993, Macri *et al* 1993). This may be explained by differentially controlled transcription of hCG α- and β-subunits (Jameson and Hollenberg 1993) with a relative excess of free β-subunit present in the first trimester (Cole *et al* 1984).

Similarly, it is possible that the forms or molecular sizes of inhibin in maternal sera in normal and Down's syndrome pregnancies may differ and that these differences are gestation dependant. Thus, assays for different immunoreactive inhibin species might have different sensitivities in Down's syndrome detection at different stages of pregnancy. Indeed, the finding of a lack of correlation of results between the two assays suggests that the two assays used in this study are detecting different inhibin species in these pregnancy samples. Interestingly, a similar disassociation of the two assays in serum samples from non-pregnant women was not observed (unpublished data) and so the current data may reflect changes in inhibin secretion specific to pregnancy. In this regard, it was reported recently that α_2-macroglobulin binds inhibin in plasma (Vaughan and Vale 1993), altering immunoreactive detectable levels by masking different epitopes. Levels of α_2-macroglobulin rise by approximately 30% in pregnancy (Studd et al 1970) and it is conceivable that this could differentially interfere with antigen-antibody binding in one or other of these two assays. Thus, the remaining uncertainty about which inhibin proteins are being detected in pregnancy and whether the placenta of a Down's syndrome pregnancy secretes different inhibins from a chromosomally normal placenta merits the evaluation of other inhibin assays in the detection of Down's syndrome in both the first and second trimesters of pregnancy. In particular, it would be important to assess assays specific for dimeric inhibin or the inhibin β-subunit.

Chapter Five

Inhibin-A: a Second Trimester Marker of Down's Syndrome

5.1 Introduction

Despite the disappointing data from the studies of immunoreactive inhibins as a prenatal marker of Down's syndrome in the first (*Chapter Four* , van Lith *et al* 1994), and second trimesters (van Lith *et al* 1992, Spencer *et al* 1993, Cuckle *et al* 1994) of pregnancy, overall the second trimester data remained encouraging. Indeed, the knowledge that the inhibin immunoassays available at that time were non-specific, able to detect monomeric inhibin α-subunit in addition to dimeric inhibins (Schneyer *et al* 1990), and that two different assays appeared to detect different inhibin moieties in pregnancy sera (*Chapter Four*) offered the prospect that of the different forms of inhibins circulating in pregnancy only selected ones were elevated in association with Down's syndrome. If it was possible to specifically detect these forms, eliminating the "background noise" of the other forms, then the performance of inhibin as a marker could be significantly improved. Of course this possibility depended on the development of novel detection methods for inhibin.

In 1991 Groome and his colleagues first described the development of a new two site assay that utilised monoclonal antibodies, one each directed against the inhibin α-subunit and the inhibin β_A-subunit (Knight *et al* 1991, Groome and O'Brien 1993). Subsequently, modifications to the assay (Knight and Muttukrishna 1994), as described in detail in *Chapter Two*, afforded a sensitivity sufficient to detect inhibin-A (α-β_A) in human sera, for which it was validated (Groome *et al* 1994). The studies related in this chapter describe the application of this specific assay to maternal sera from chromosomally normal and Down's syndrome pregnancies in the second trimester.

5.2 Materials and Methods

5.2.1 Study one.

5.2.1.1 Serum samples

In south-east Scotland prenatal screening for Down's syndrome and neural tube defects is offered to all women booking antenatally with a singleton pregnancy. An aliquot of each serum sample collected for this programme is routinely stored at -20°C. Of the known Down's affected pregnancies during 1992-1993 there were twenty-one women from whom stored serum was available and these sera were retrieved. All these women had been at 15-17 completed weeks of pregnancy at the time of

sampling, as calculated from a first or early second trimester ultrasound scan. Fourteen of the 21 (67%) had been detected prenatally by the screening programme. Samples from 150 control women with a chromosomally normal pregnancy matched for gestation (within one week as determined by ultrasound) and duration of storage, were identified and retrieved. Forty-five of the control samples were at 15 completed weeks, 55 at 16 completed weeks and 50 at 17 completed weeks of pregnancy.

5.2.1.2 Inhibin-A assay

Format I *(Chapter Two)* of the assay was used in this study, employing the recombinant 32kDa human inhibin-A preparation (Genentech Inc, CA, USA) as the calibrating standard. The sensitivity of the assay was 8 pg/mL and the intraplate and interplate coefficients of variation were 2.5% and 7.0% respectively. Thus, in the context of *Chapters Two* and *Nine* the assay format used in this study was I-RH.

5.2.1.3 Statistical analyses

Statistical analyses were performed using the software package Statview 4.1 (Abacus Inc. Berkeley, CA, USA). Inhibin levels in the control samples at each separate week of gestation were compared using the Mann-Whitney U test. Since a significant difference across gestation of pregnancy was detected in the controls, the Down's samples and controls were expressed as multiples of the normal median (MoM) to allow comparison between the two groups. Each MoM, for both control and Down's samples, was calculated from the normal median of the respective gestation.

5.2.2 Study two

5.2.2.1 Serum samples

Aliquots of selected maternal serum samples that had been collected as part of the West of Scotland prenatal screening programme for Down's syndrome and neural tube defects between 1987 and 1994 were retrieved from storage. In total, 528 serum samples were available for study from this programme, of which 254 samples had been collected in the first trimester (7 to 14 weeks of pregnancy) and 274 in the second trimester (15 to 18 weeks of pregnancy). While the inhibin-A results for all 438 chromosomally normal samples are reported in this chapter for clarity, for the trisomic pregnancies only the analyses and data relating to the second trimester samples will be discussed here. Therefore, of the 274 second trimester samples, 202 were from a chromosomally normal pregnancy, 44 from a trisomy 21 (Down's syndrome) pregnancy and 28 from a trisomy 18 (Edward's syndrome) pregnancy. Of the Down's syndrome samples, 35 had been previously reported in a prospective study (Crossley *et al* 1994). The control samples had been chosen, from archival records, to match the

trisomic samples for gestation and duration of storage. Gestation had been calculated, for all samples, in completed weeks of pregnancy from certain menstrual dates or from an ultrasound scan. The identification and retrieval of all samples was performed by Dr Jenny Crossley and Dr David Aitken, Duncan Guthrie Institute, Glasgow as part of a collaborative project.

5.2.2.2 Assays

Inhibin-A was measured in Edinburgh using format I-RH of the inhibin-A ELISA, as described in *Chapter Two*. The sensitivity of the assay was 8 pg/mL and the intraplate and interplate coefficients of variation were 2.5% and 7.0%, respectively.

Other analyte assays had been previously performed in Glasgow. Intact hCG and AFP had been measured prospectively, as part of the routine screening programme (Crossley *et al* 1994), using a commercial immunoradiometric assay (MAIAClone, Serono, Rome, Italy) and an in-house immunoradiometric assay (Aitken *et al* 1993), respectively. Samples were analysed for free β-hCG retrospectively, using an ELISA as previously described (Macri *et al* 1993).

All samples were assayed for all analytes blinded to whether they were from a normal or a trisomic pregnancy.

5.2.2.3 Statistical analyses

Statistical analysis of the data was performed by Dr Jenny Crossley, Duncan Guthrie Institute, Glasgow.

To allow comparisons between normal controls and trisomic pregnancies, values of all analytes were converted to multiples of the median (MoM). The median inhibin-A level for each completed week of gestation was calculated from the 438 chromosomally normal controls and levels in both the control and trisomic samples thereafter converted to MoMs using these median values. MoMs for intact hCG, free β-hCG and AFP were calculated from median normal values previously calculated from the much larger dataset from which the samples reported here had been derived.

Goodness of fit to log gaussian distributions for each marker in both the control and trisomic samples was assessed by probability plot and the Kolmogorov-Smirnov test. Measures of distribution for each marker, in both normal and trisomic samples, were calculated by using the $\log_{10}$ of the median as the mean and the difference between the 10^{th} and 90^{th} percentiles in logs, divided by 2.56 as the standard deviation (Cuckle *et al* 1987).

For the Down's syndrome samples, detection and false positive rates were calculated with gaussian models of the distribution of likelihood ratios and the age distribution of pregnancies in the population from which the samples had been derived, as described in detail elsewhere (Reynolds and Penny 1990, Zeitune *et al* 1991, Aitken *et al* 1996).

5.3 Results

5.3.1 Study one

Table 5.1 (page 70) shows the median (95% CI) maternal serum levels of dimeric inhibin A in the chromosomally normal pregnancies. The level at 17 weeks gestation was significantly lower than that at 16 weeks ($p<0.02$, Mann-Whitney U test). There were no other significant differences.

In all of the Down's syndrome samples the dimeric inhibin levels were above the normal median (Figure 5.1, page 71). The median (95% CI) MoM was 2.6 (2.25-3.57), significantly higher than controls ($p<0.0001$, Mann-Whitney U test). For a given false positive rate (FPR) of 5.3% (8/150), 62% (13/21) of the Down's syndrome samples would have be detected at a cut off MoM of 2.2. Table 5.2 (page 70) shows the detection rates and corresponding FPRs at different arbitrary MoMs.

The inhibin data fitted a log Gaussian frequency distribution with standard deviation of $\log_{10}$(inhibin-A) of 0.27 in the controls and 0.20 in the Down's syndrome cases. The mean $\log_{10}$(inhibin-A) in the Down's syndrome cases was 0.41.

Of the seven Down's samples that had been undetected by the routine screening programme two had dimeric inhibin A levels above the arbitrary threshold of 2.2 MoM while three Down's samples with levels below this arbitrary detection limit had been detected by screening (table 5.3, page 72).

Table 5.1

Median (95% CI) maternal serum dimeric inhibin A levels in 150 chromosomally normal singleton pregnancies at 15 - 17 weeks gestation.

Gestation (weeks)	Number of samples	Inhibin-A (pg/mL)
15	45	220.4 (201.5 - 273.4)
16	55	246.5 (235.4 - 298.5)
17	50	182.0 (178.5 - 235.9)

Table 5.2

Number (percentage) of affected and unaffected pregnancies with a given MoM above different various levels.

MoM	Number (%) of Affected Pregnancies	Number (%) of Unaffected Pregnancies
0.5	21 (100)	135 (90)
1.0	21 (100)	76 (51)
1.5	21 (100)	28 (19)
2.0	14 (67)	11 (7)
2.5	11 (52)	4 (3)
3.0	7 (33)	1 (1)
3.5	6 (29)	0 (0)
4.0	6 (29)	0 (0)
4.5	4 (19)	0 (0)

Figure 5.1

Median maternal serum inhibin-A MoMs (10th - 90th percentiles) in 150 chromosomally normal pregnancies with levels from 21 individual Down's syndrome pregnancies.

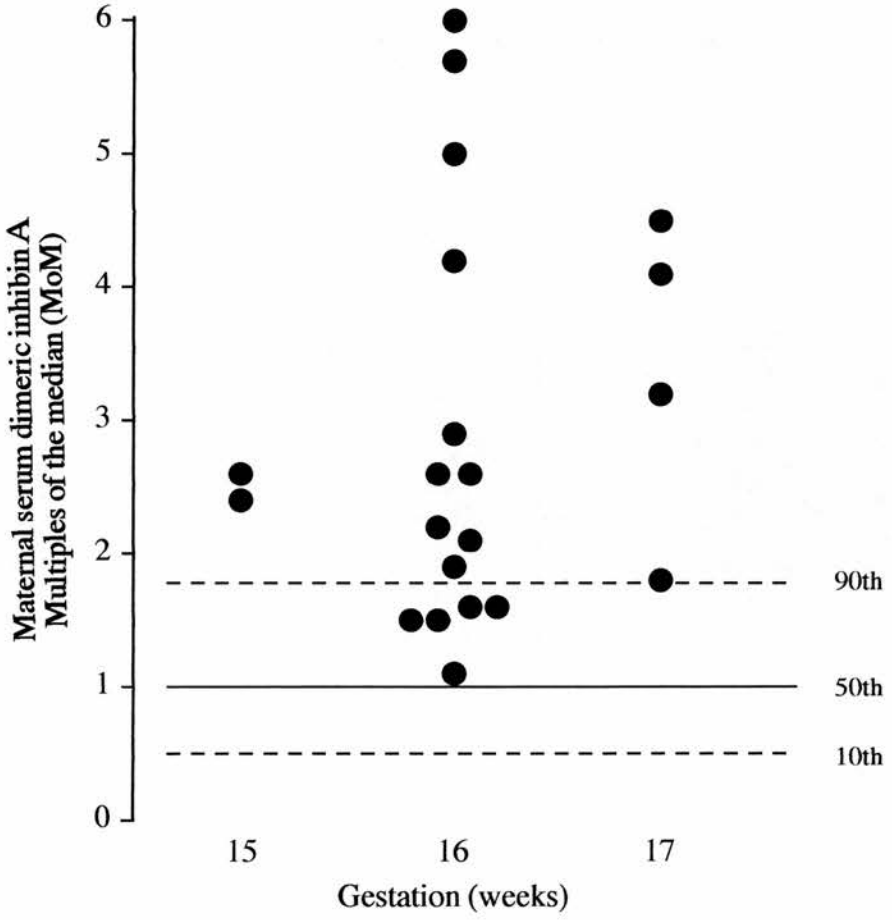


Table 5.3

Maternal serum inhibin-A levels (MoM) in 21 Down's affected pregnancies compared with the result from routine screening based upon maternal age/AFP/hCG.

Inhibin-A (MoM)	Detected/Undetected by Standard Screening	Inhibin-A (MoM)	Detected/Undetected by Standard Screening
6.0	undetected	2.4	detected
5.7	detected	2.2	detected
5.0	detected	2.1	detected
4.5	detected	1.9	undetected
4.2	detected	1.8	undetected
4.1	detected	1.6	undetected
3.2	detected	1.6	detected
2.9	undetected	1.5	undetected
2.6	detected	1.5	detected
2.6	detected	1.1	undetected
2.6	detected		

5.3.2 Study two

Figure 5.2 (page 73) shows the inhibin-A levels in the normal control samples across the complete control sample set from 7 to 18 weeks of pregnancy. Levels fall from a peak at 8-9 weeks of pregnancy to a plateau at 15 weeks.

Table 5.4 (page 74) shows the levels of inhibin-A, intact hCG, free β-hCG and AFP in the control and Down's syndrome samples. In the Down's syndrome samples inhibin-A, intact hCG and free β-hCG were significantly elevated and AFP was significantly decreased (Mann-Whitney U test). The extent of the inhibin-A increase was favourable in comparison to both intact hCG and free β-hCG.

Figure 5.2

Median (10^{th}-90^{th} percentiles) inhibin-A levels (pg/mL) in 438 chromosomally normal singleton pregnancies from 7 to 18 weeks of pregnancy.

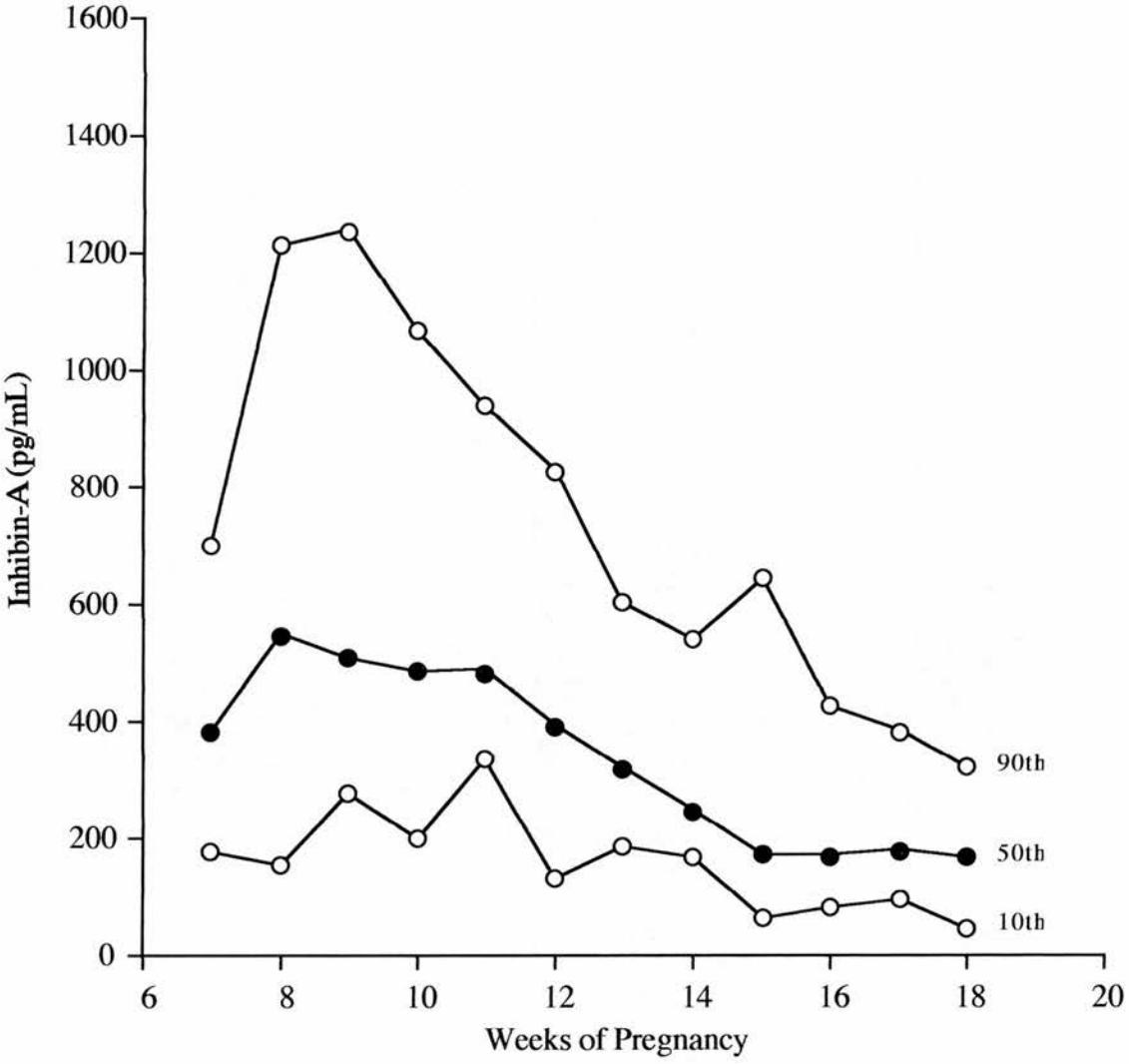


Table 5.4

Levels of serum markers, expressed as MoMs, in control and Down's syndrome pregnancies at 15-18 weeks of gestation.

Marker	**Controls**		**Down's Syndrome**		**p-value**
	No. of samples	MoM	No. of samples	MoM	
Inhibin-A	202	1.00	44	2.24	<0.001
Intact hCG	202	1.03	44	2.04	<0.001
Free β-hCG	142	1.03	44	2.05	<0.001
AFP	202	0.95	44	0.74	<0.001

The goodness of fit for inhibin-A was optimum to a log-gaussian distribution, as assessed by the Kolmogorov-Smirnov test, and subsequent analyses were performed assuming log normality for the inhibin-A results. It was known from previous studies (Zeitune *et al* 1991, Crossley *et al* 1992, Spencer *et al* 1992) that AFP, intact hCG and free β-hCG fitted log gaussian distributions. Table 5.5 shows the correlation statistics between the different markers, including maternal age. Inhibin-A significantly correlated with intact hCG levels in both the control and Down's syndrome samples and with AFP in the controls only. Inhibin-A was not correlated with free β-hCG nor with maternal age, in either the controls or the affected cases.

Table 5.5

Coefficients of correlation (r) between prenatal markers of Down's syndrome in 202 chromosomally normal control and 44 Down's syndrome pregnancies at 15-18 completed weeks of pregnancy.

	Controls		**Down's cases**	
	r	p-value	r	p-value
Log (inhibin MoM) v Log (intact hCG MoM)	0.27	<0.001	0.39	0.008
Log (inhibin MoM) v Log (FβhCG MoM)	0.15	0.107	0.23	0.134
Log (inhibin MoM) v Log (AFP MoM)	0.24	0.001	0.24	0.110
Log (inhibin MoM) v maternal age	-0.03	0.690	0.25	0.098

The distribution parameters of the analytes are described below in table 5.6.

Table 5.6
Means and standard deviations (SD) of log gaussian distributions for analytes in 202 chromosomally normal control and 44 Down's syndrome pregnancies at 15-18 completed weeks of pregnancy.

Analyte	**Controls**		**Down's cases**	
	mean	SD	mean	SD
Inhibin-A	0.0000	0.2967	0.3502	0.3521
Intact hCG	0.0128	0.2196	0.3086	0.2064
Free βhCG	0.0128	0.2609	0.3188	0.3061
AFP	-0.0223	0.1609	-0.1337	0.1805

Using the distribution statistics for each marker in both the normal controls and the Down's syndrome cases, the detection rates, for a fixed 5% FPR, were calculated for different marker combinations. In this dataset, the optimum marker combination, in terms of the detection rate, was maternal age/AFP/free β-hCG/inhibin-A which afforded a detection rate of 75% for a FPR of 5% (table 5.7).

Table 5.7
Detection rates (95% CI), at a 5% false positive rate, for Down's syndrome for various marker combinations.

Combination	DR (%)	95% CI
AFP / intact hCG / age	54	38 - 69
AFP / FβhCG / age	53	37 - 68
AFP / inhibin-A / age	57	41 - 72
FβhCG / inhibin-A / age	68	52 - 81
AFP / intact hCG / inhibin-A / age	72	57 - 84
AFP / FβhCG / inhibin-A / age	75	60 - 87

Expressed as MoMs, the inhibin-A level in the trisomy 18 samples and controls was 0.84 and 1.0, respectively, not significantly different. in contrast, the intact hCG level in the same samples was 0.3 and 1.03, respectively ($p<0.001$, Mann-Whitney U test).

5.4 Discussion and conclusions

Maternal serum inhibin levels have previously been shown to be elevated in Down's affected pregnancies in the second (Van Lith *et al* 1992, Spencer *et al* 1993, Cuckle *et al* 1994) but not first trimester (*Chapter Four*, van Lith *et al* 1995). However, in the three second trimester studies the variation in both the absolute inhibin levels in the controls and in the degree of elevation in the Down's cases reported and the strong association between inhibin and hCG levels suggested that immunoreactive inhibin, as detected, was unlikely to prove a valuable prenatal marker of Down's syndrome (Cuckle *et al* 1994).

Nonetheless, there are a number of possible inhibin species circulating in peripheral blood including the two 31kDa dimeric forms (inhibin-A and inhibin-B), larger dimeric forms (58-73kDa), large free precursor α-subunits and the free processed α-subunit (Robertson *et al* 1995). By the nature of the antibodies employed, the immunoreactive assays used in the above studies (van Lith *et al* 1992, Spencer *et al* 1993, Cuckle *et al* 1994, *Chapter Four*, van Lith *et al* 1995) are unable to specifically detect intact dimeric inhibin A but probably also detect free inhibin α-subunits and the related pro-αC and pro-αC-pro-αN fractions (Schneyer *et al* 1990). It is possible that this non-specificity may account for the very heterogeneity of the results that has caused some concern (Cuckle et al 1995). In *Chapter Four* however, it was reported that two immunoassays, both utilising anti inhibin α-subunit antibodies, appeared to recognise different inhibin species in the same pregnancy samples, possibly due to differing antibody affinities for different species. Further, serum factors that are known to interfere with immunoreactive inhibin detection (Vaughan and Vale 1993) increase in pregnancy (Studd *et al* 1970). It is clear therefore, that the usefulness of inhibin in prenatal screening for Down's syndrome may crucially depend on which inhibin species are being detected and thus on the antibodies utilised in the assay(s) applied. The studies reported here represent the first application of an assay specific for inhibin-A to the detection of Down's syndrome in the second trimester. *Chapter Six* relates similarly novel data for the first trimester.

Despite the wide distribution of non-specific immunoreactive inhibin noted previously, using the Medgenix inhibin assay van Lith and colleagues (1992) reported a preliminary detection rate of 40% with a false positive rate of 5% and a median MoM (95% CI) of 1.3 (0.9 - 1.9) for inhibin alone across a gestational range of 14-18 weeks. In Study One reported here the corresponding detection rate of 62% for a 5.3% FPR and median MoM (95% CI) of 2.6 (2.25-3.57) for inhibin A at 15-17 weeks gestation would appear very favourable to non-specific immunoreactive inhibin. Also, in the same study the standard deviations of $\log_{10}$(inhibin-A) in both the controls and the Down's cases show that inhibin-A levels are less widely distributed than the non-specific immunoreactive inhibin previously reported. This phenomenon could explain the improved detection performance of intact dimeric inhibin-A relative to immunoreactive inhibin. It is also possible that only some of the circulating inhibin species are discriminatory with regard to Down's syndrome. If the less specific assays were detecting some of the non-discriminatory inhibin species, undetected by the dimeric assay, then poorer detection performance would be observed. In support of this suggestion, Illingworth and his colleagues (1996a) recently showed that pro-αC containing inhibins circulate in early pregnancy, probably reflecting monomeric α-subunit rather than dimers containing this precursor fragment (Robertson *et al* 1997), and that levels of pro-αC inhibins in Down's syndrome are not significantly different from normal (Wallace, unpublished data). Further, in collaboration with Howard Cuckle, Leeds University, the author has assayed inhibin-A in the sample set on which immunoreactive inhibin levels were previously reported (Cuckle *et al* 1994). Instead of the median MoM of 1.3 observed for immunoreactive inhibin, the median inhibin-A level in the Down's syndrome cases was 1.6, significantly higher (Cuckle *et al* 1995), suggesting preferred detection of discerning inhibin forms by the inhibin-A assay.

In Study One it was also encouraging to observe that inhibin-A detected Down's syndrome cases that had previously screened negative with traditional markers. This suggests that this inhibin species is not associated with hCG and so may have a useful role as an adjuvant marker in the second trimester, increasing detection rates. This observation is quite different from the tight correlations previously observed between hCG and immunoreactive inhibin (Spencer *et al* 1993, Cuckle *et al* 1994). Study Two was therefore undertaken to expand upon these initially exciting observations and to formally assess the relationships between inhibin-A and existing prenatal serum markers.

Confirming the results from Study One, Study Two demonstrated significantly higher inhibin-A levels in Down's syndrome compared to normal pregnancy. Indeed, while the extent of this elevation was not as high as observed in Study One, it was still higher than either intact hCG or free β-hCG. Importantly, Study Two showed that there was no significant association between inhibin-A and free β-hCG levels. This would suggest that these two markers would have additional power in the detection of affected cases. Interestingly, a more recent report did not confirm this lack of association (Spencer *et al* 1996) and the more limited additional detection afforded by inhibin-A in that series reflects that observation. Nonetheless, in this series inhibin-A added an additional 20% detection to existing marker combinations, representing a significant improvement in screening performance. In other, more recent series, similar improvements in detection rates have been reported (Wald *et al* 1996, Cuckle *et al* 1996) with a meta-analysis of all data, excepting those of Spencer and his colleagues (1996), suggesting that inhibin-A will improve the detection rate of Down's syndrome by approximately 10% for a fixed 5% FPR (Cuckle *et al* 1996).

Intriguingly, while the maternal serum level of inhibin-A in trisomy 18 samples was, on average, slightly reduced it was not significantly so. This was a surprising finding considering the hCG level was profoundly low, as has been reported by others previously (Ozturk *et al* 1990). Indeed, this observation represents the first disassociation of inhibin and hCG levels in maternal serum and may provide some new insights into the differential control of the secretion of these proteins.

The normal range data derived from Study Two and displayed in figure 5.2, page 73, extends back into the first trimester, further than any previous data reported in this thesis and usefully confirms the biphasic ontogeny described at length in *Chapter Three* and elsewhere (Muttukrishna *et al* 1995, Illingworth *et al* 1996). Furthermore, the plateauing levels at 15-18 weeks is a useful observation. The levels of all serum markers change with gestation, hence the requirement for expressing levels as MoMs allowing comparisons across gestations. Therefore, inaccuracies in gestational dating impact upon the performance of screening and will either decrease the detection rate, if the FPR is kept constant, or increase the FPR if the detection rate is maintained (RCOG 1993). This effect is especially critical because in 10-45% of pregnancies women are uncertain of their menstrual dates (Campbell *et al* 1985) and even in those women with certain dates, in a third there will be an error in excess of 7 days (Geirsson 1991). However, inhibin-A levels change minimally across the window of 15-18 weeks and so gestation-associated errors would be minimal with this marker, a most useful attribute.

In conclusion, the studies reported in this chapter have shown that inhibin-A is significantly elevated in maternal serum in Down's syndrome pregnancies in the second trimester and that, when combined with existing markers, inhibin-A will improve prenatal screening detection rates. This represents a significant development in Down's syndrome screening and the eventual application of inhibin-A now depends upon cost-benefit analyses of the new marker combinations (Wald *et al* 1996).

Chapter Six

Inhibin-A: a First Trimester Marker of Down's Syndrome

6.1 Introduction

While second trimester maternal serum screening for Down's syndrome is certainly highly effective (RCOG 1993), it is generally accepted that it would be desirable to offer similar screening earlier in pregnancy. This approach would afford women, and their partners, the opportunity of earlier diagnosis, if indicated, and earlier termination of pregnancy, if appropriate. It is assumed, although not tested, that in cases of fetal abnormality, such as aneuploidy, a surgical termination of pregnancy performed under general anaesthesia as a day-case is less traumatic psychologically than a prostaglandin termination which requires the women to "deliver" her fetus vaginally while conscious. In support of this assumption is the recent finding that women undergoing early pregnancy termination for pyschosocial reasons appear to prefer termination performed under general anaesthesia to medical termination which essentially induces a "miscarriage" (Cameron *et al* 1996). Accepting these arguments, which are very plausible but remain to be explored objectively, it is clearly important to be able to offer women early screening if early termination is to be an option. There are however, other considerations.

Approximately two thirds of Down's syndrome pregnancies will be lost spontaneously before delivery at term, with as many as 15-30% being lost between 10 and 16 weeks (Macintosh *et al* 1996, Halliday *et al* 1995). This is of critical importance because some affected pregnancies that are viable at 10-14 weeks and so accessible to first trimester screening may miscarry before second trimester serum screening is performed. Indeed, abnormal screening may preferentially identify pregnancies destined to be lost (Hyett *et al* 1996, Benn *et al* 1996). It is thus conceivable that first trimester screening would expose many women unnecessarily to significant distress and anguish over choices regarding termination (Wallace 1997), representing a real disadvantage of earlier testing. Furthermore, current second trimester serum screening is for aneuploidy (trisomies 21 and 18) and neural tube defects, whereas first trimester serum screening would not be a sensitive method of screening for neural tube defects (Shalev *et al* 1992, Wald *et al* 1993) and later screening would still have to be performed.

Nonetheless, the feasibility of first trimester serum screening for Down's syndrome has received much attention, as reviewed recently by Macintosh and Chard (1993). Of the various serum markers assessed free β-hCG, AFP and pregnancy associated plasma protein A (PAPP-A) have been the most fully evaluated to date (Macintosh and Chard 1993). Maternal serum fβhCG is significantly elevated in Down's syndrome

pregnancies (Spencer *et al* 1992, Macri *et al* 1993) while AFP (Brambati *et al* 1986, Sciosca *et al* 1987, Crandall *et al* 1991, Cuckle *et al* 1988, Brock *et al* 1990, Aitken *et al* 1993) and PAPP-A (Brambati *et al* 1991, Brambati *et al* 1993, Muller *et al* 1993, Hurley *et al* 1993) are significantly lowered. In combination with maternal age, a first trimester serum screening programme based upon these markers might offer a detection rate of approximately 60% for a 5% false positive rate (Aitken *et al* 1993, Krantz *et al* 1996), almost as good as is achieved by the optimum marker combination in the second trimester.

As discussed in *Chapters Four* and *Five*, the finding that, within the same samples, there was no correlation between maternal serum immunoreactive inhibin levels detected by two different assays, an observation previously not made for menstrual cycle sera, and that immunoreactive inhibin levels were elevated in the second trimester, merited further assessment of specific inhibins as markers of Down's syndrome. With respect to first trimester screening, the small study examining immunoreactive inhibin levels described earlier (*Chapter Four*) did suggest that one of the assays used (NICHD) may have detected differences in inhibin levels between controls and Down's syndrome pregnancies at eleven weeks gestation. Thus, when an assay specific for inhibin-A and sensitive enough to detect levels in sera became available (Groome *et al* 1994) the potential of this inhibin dimer as a first trimester marker of Down's syndrome was examined. Importantly, the studies detailed in this chapter were performed contemporaneously with those in *Chapter Five*, as is evident from the similar evolution of assay methodology used in the different studies, as explained in *Chapter Two* and examined again in *Chapter Seven*.

6.2 Materials and Methods

6.2.1 Study one.

6.2.1.1 Serum samples

As described in *Chapter Four* (4.2.1, page 58) in Lothian sera are routinely stored from first trimester booking blood serology. From these records, of all known Down's affected pregnancies twenty-three women (mean age 32.1 years, range 22-44 years) were identified retrospectively allowing their stored serum to be retrieved. The sera from eight of these women had been collected at 11 completed weeks of gestation, eight at 12 completed weeks and seven at 13 completed weeks, calculated from ultrasound scans performed on the day of sampling. In 11 of these women immunoreactive inhibin had been previously measured (*Chapter Four*). For each

Down's affected sample four control women, matched for gestation (ultrasound determined) and duration of storage, were identified and their samples retrieved (mean age 27.7 years, range 19-38 years). Three control samples, one each matched for three different 11 week Down's samples, were unable to be used due to insufficient sample volume, making 89 control samples available for assay.

6.2.1.2 Assays

Inhibin-A assay format I-RH was utilised in this study. The sensitivity of the assay was
8 pg/ml and the intraplate and interplate coefficients of variation were 2.5% and 7.0%, respectively.

Pregnancy associated plasma protein A (PAPP-A) and free β-hCG were measured by a commercial assay in development by the laboratory of the relevant company (Johnson and Johnson, Cardiff, UK). This facility was generously made available by Dr Chris Davies of Johnson and Johnson (see *Acknowledgments*, page viii).

All assays were performed blinded to whether the sample was from a normal or Down's syndrome pregnancy.

6.2.1.3 Statistical analyses

Statistical analyses were performed using the software package Statview 4.1 (Abacus Inc. Berkeley, CA, USA). Inhibin levels in the control samples at each separate week of gestation were compared using the Mann-Whitney U test. Since a significant difference across gestation of pregnancy was detected in the controls, the Down's samples and controls were expressed as multiples of the normal median (MoM) to allow comparison between the two groups. Each MoM, for both control and Down's samples, was calculated from the normal median of the respective gestation.

Similar analyses were performed for both PAPP-A and free β-hCG. Relationships between levels of the three proteins were also explored using simple correlation analysis of log transformed data.

6.2.2 Study two.

6.2.2.1 Samples, assays and analyses

This study is the first trimester component of Study Two in *Chapter Five*, page 67. As detailed in that chapter, there were 254 first trimester samples from 7-14 completed weeks of gestation. Four of these samples were from trisomy 18 pregnancies and will not be discussed further (see page 76). Of the remaining 250 samples, 236 were from

a chromosomally normal singleton pregnancy and 14 from a trisomy 21 pregnancy. The assays and statistical analyses are as described previously (page 68).

6.2.3 Study three

6.2.3.1 Serum samples

All the samples for this study were generously supplied by Dr Penny Noble for a collaborative project with Professor Kypros Nicolaides at the Harris Birthright Research Centre for Fetal Medicine, London (see *Acknowledgments*, page viii). In each case, blood had been collected immediately before an ultrasound examination at the Harris Birthright Research Centre for Fetal Medicine and stored at 4°C until assayed for free β-hCG the following day. Thereafter the sera were stored at -20°C, for up to 12 months, until analysed, in Edinburgh, for inhibin-A content. Seventy-six samples from Down's syndrome pregnancies and 800 with normal pregnancies were available for analysis. In each case, the women were self referrals to the Harris Birthright Research Centre for Fetal Medicine for screening for Down's syndrome. Gestation (in completed weeks) was calculated from the ultrasound scan performed prior to sampling.

Ethical approval had been granted for this study by the King's College Hospital ethics committee.

6.2.3.2 Assays

Inhibin-A was assayed using a hybrid format not detailed elsewhere. Indeed, this study is the only one to have been performed with this format. Sample preparation was similar to format I (ie no boiling pre-treatment) but detection of the signal was afforded with the simple alkaline phosphatase substrate, pNPP (KPL, Maryland, USA). The standard preparation used was the recombinant product from Genentech. As detailed in *Chapter Seven* the method of detection does not affect the results obtained, provided levels remain within levels of detection. This assay is therefore most like format I-RH than any other. The sensitivity of the assay was 39 pg/ml and the intraplate and interplate coefficients of variation were 4.3% and 5.6%, respectively.

Free β-hCG was measured, in London, using a commercial immunoradiometric assay (CIS, Paris, France). The sensitivity of the assay was 0.15ng/mL and the intra-assay and inter-assay coefficients of variation were 3.1% and 5.7%, respectively.

6.2.3.3 Statistical analyses

Statistical analyses were performed using the software package Statview 4.1 (Abacus Inc. Berkeley, CA, USA). Inhibin levels in the control samples at each separate week

of gestation were compared using the Mann-Whitney U test. Since a significant difference across gestation of pregnancy was detected in the controls, the Down's samples and controls were expressed as multiples of the normal median (MoM) to allow comparison between the two groups. Each MoM, for both control and Down's samples, was calculated from the normal median of the respective gestation.

Similar analyses were performed for free β-hCG. Relationships between levels of the three proteins were explored using simple correlation analysis of log transformed data.

6.3 Results

6.3.1 Study one

6.3.1.1 Inhibin-A results

Table 6.1 (below) shows the inhibin-A levels in the control samples. There was no significant difference between the levels at 11 and 12 weeks (p=0.054, Mann-Whitney U test) but the mean level at 13 weeks was significantly lower than that at 11 weeks (p=0.0004, Mann-Whitney U test) though not 12 weeks (p=0.055).

Figure 6.1 (page 86) shows inhibin-A levels, expressed as MoMs, in the 23 Down's syndrome cases. The median (95% CI) MoM for the Down's syndrome samples was 2.46 (2.11-3.26), significantly higher than the controls (p<0.0001 v controls, Mann Whitney U test). Table 6.2 (page 87) displays the number of affected and unaffected pregnancies at different MoMs. In this series, for a given false positive rate of 4% (4/89), 65% (15/23) of the Down's cases would have been detected.

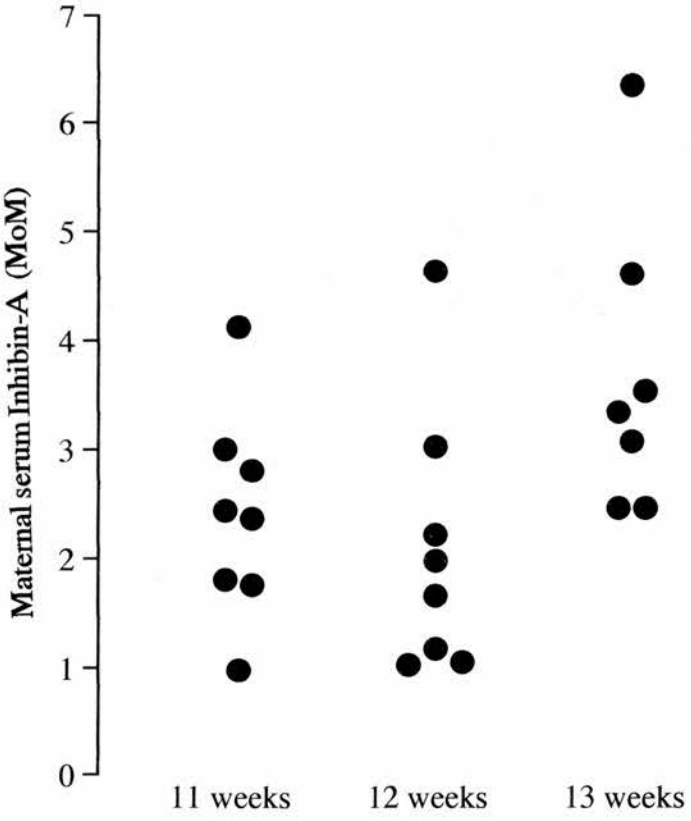
Maternal serum Inhibin-A was not associated with maternal age (r=1.0, p=0.49).

Table 6.1
Median (10th-90th percentiles) maternal serum inhibin-A in 89 chromosomally normal singleton pregnancies at 11-13 weeks of pregnancy.

Gestation (weeks)	Number of samples	Inhibin-A (pg/mL)
11	29	361.8 (250.8-524.6)
12	32	279.7 (183.5-564.5)
13	28	221.0 (120.9-453.8)

Forty-four control and eleven Down's syndrome samples were available to study associations between inhibin-A levels and immunoreactive inhibin levels. In the control samples, there was a significant association between $\log_{10}$(inhibin-A MoM) and $\log_{10}$(NICHD inhibin MoM) (r=0.35, p=0.03) but no association between $\log_{10}$(inhibin-A MoM) and $\log_{10}$(Medgenix inhibin MoM) (r=0.17, p=0.29). In the Down's syndrome samples there were no significant associations (r=0.28, p=0.4 and r=0.45, p=0.17 for $\log_{10}$(inhibin-A MoM) v $\log_{10}$(NICHD inhibin MoM) and $\log_{10}$(inhibin-A MoM) v $\log_{10}$(Medgenix inhibin MoM), respectively).

Figure 6.1.
Maternal serum inhibin-A levels (MoMs) in 23 individual Down's syndrome pregnancies.



6.3.1.2 PAPP-A and free β-hCG results

Table 6.3 (page 87) shows the median (95% CI) PAPP-A and free β-hCG levels in the control sera. Levels of PAPP-A increased across the gestations studies while free β-hCG levels decreased. Expressed as MoMs (95% CI), in the 23 Down's syndrome samples the PAPP-A level was 0.89 (0.71-1.27) MoM, not significantly different

from normal ($p>0.05$, Mann-Whitney U test) and the free β-hCG level was 1.89 (1.72-2.67), significantly elevated ($p<0.0001$, Mann-Whitney U test).

Table 6.2.

Number (percentage) of affected and unaffected pregnancies with a given MoM above different arbitrary levels.

MoM	Number (%) of Affected Pregnancies	Number (%) of Unaffected Pregnancies
0.5	23 (100)	86 (97)
1.0	22 (96)	42 (47)
1.5	19 (83)	18 (20)
2.0	15 (65)	6 (7)
2.5	10 (43)	1 (1)
3.0	9 (39)	1 (1)
3.5	5 (22)	0 (0)
4.0	4 (17)	0 (0)
4.5	3 (13)	0 (0)

Table 6.3

Median (10th-90th percentiles) maternal serum inhibin-A in 89 chromosomally normal singleton pregnancies at 11-13 weeks of pregnancy.

Gestation (weeks)	Number of samples	PAPP-A (iu/mL)	Fβ-hCG (iu/L)
11	29	2959 (912-4922)	22.9 (10.3-34.9)
12	32	3315 (1306-5274)	19.6 (7.3-29.3)
13	28	4606.0 (1753-5539)	15.4 (5.7-21.6)

6.3.1.3 Correlation between inhibin-A, PAPP-A and free β-hCG.

In both the control and Down's syndrome samples $\log_{10}$(inhibin-A MoM) was significantly correlated with $\log_{10}$(free β-hCG MoM) ($r=0.61$, $p<0.001$ for the controls and $r=0.65$, $p<0.001$ for the Down's syndrome cases). There were no other significant associations between proteins in either the controls or the Down's syndrome cases.

6.3.2 Study two

The median inhibin-A level in the normal control pregnancies is shown in figure 5.2, page 73. In the Down's syndrome pregnancies, at 7-11 weeks gestation the median inhibin-A level was not significantly different from normal but it was significantly elevated at 13-14 weeks. Free β-hCG levels in the Down's syndrome samples were elevated at all gestations (table 6.4)

Table 6.4
Levels of serum markers, expressed as MoMs, in control and Down's syndrome pregnancies at 7-14 weeks of gestation.

Marker	**Gestation**	**Controls**		**Down's**		**p-value**
		No.	MoM	No.	MoM	
Inhibin-A	7-11	148	1.00	8	0.98	0.68
	13-14	88	1.00	6	2.60	0.002
Intact hCG	7-11	148	1.08	8	0.95	<0.001
	13-14	88	0.93	6	1.19	0.09
Free β-hCG	7-11	140	1.00	8	1.79	<0.001
	13-14	88	0.94	6	2.15	0.002
AFP	7-11	148	0.99	8	0.7	0.10
	13-14	88	0.96	6	0.59	0.02

6.3.3 Study three

6.3.3.1 Inhibin-A results

Table 6.5 (page 89) shows the median levels (10th-90th percentiles) of inhibin-A in the 800 chromosomally normal control samples across the gestation 10-13 weeks, inclusive. Levels fell significantly from 207 ng/mL at 10 weeks to 146.6 ng/mL at 13 weeks ($p<0.001$, Mann-Whitney U test).

In the Down's syndrome samples, the median (95% CI) inhibin-A level, expressed as MoMs, was 1.46 (1.37 - 1.85), significantly higher than in the controls ($p<0.0001$, Mann-Whitney U test). Figure 6.2 (page 90) displays the individual cases. For a given

5% false positive rate, inhibin-A alone would have detected 12.8% of the Down's syndrome cases.

Table 6.5
Median (10th-90th percentiles) maternal serum inhibin-A and free β-hCG levels in 800 chromosomally normal singleton pregnancies at 10-13 weeks of pregnancy.

Gestation (weeks)	Number of samples	inhibin-A (pg/mL)	Fβ-hCG (iu/L)
10	52	207.0 (104.3-440.6)	45.2 (21.4-113.3)
11	318	174.0 (73.2-329.9)	41.0 (18.9-99.0)
12	325	168.6 (84.1-309.7)	34.0 (16.3-78.0)
13	105	146.6 (71.3-281.1)	30.0 (14.7-69.2)

6.3.2.2 Free β-hCG results

Table 6.5 shows the levels of free β-hCG in the 800 control samples. As for inhibin-A levels fell significantly across the gestations studied from 45.2 iu/L at 10 weeks to 30.0 iu/L at 13 weeks of pregnancy ($p<0.001$, Mann-Whitney U test).

In the Down's syndrome samples, the median (95% CI) free β-hCG level was 1.91 (1.68-2.31), significantly higher than the controls ($p<0.0001$, Mann-Whitney U test; figure 6.2, page 90). For a given 5% false positive rate free β-hCG alone would have detected 28.9% of the Down's syndrome cases.

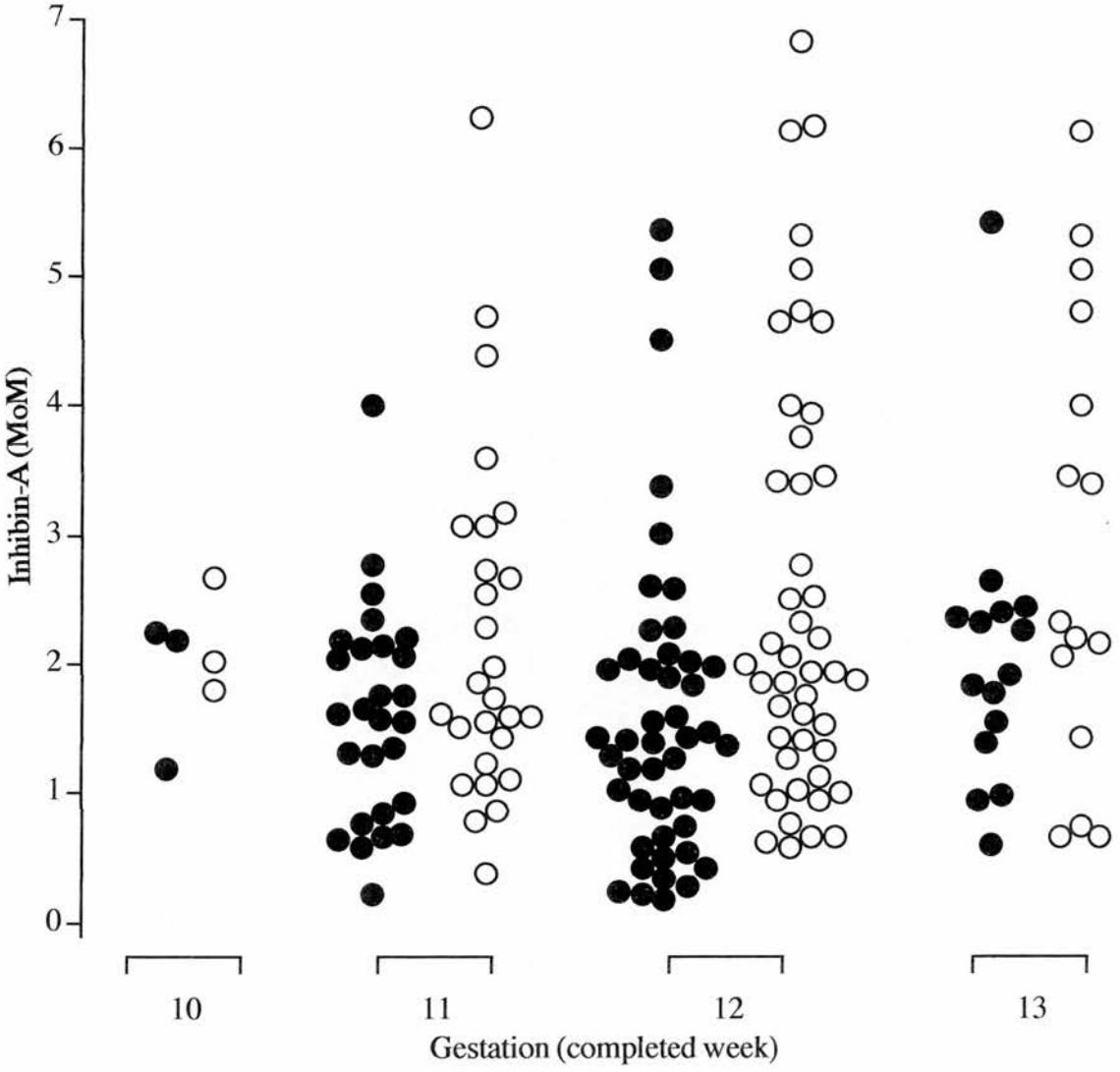
The free β-hCG levels in the Down's syndrome samples, expressed as MoMs, were significantly higher than the inhibin-A levels (figure 6.2; $p<0.0001$, paired t-test).

6.3.1.3 Correlation between inhibin-A and free β-hCG.

In both the control and Down's syndrome samples $\log_{10}$(inhibin-A MoM) was weakly but significantly correlated with $\log_{10}$(free β-hCG MoM) ($r=0.38$, $p=0.0001$ for the controls and $r=0.32$, $p=0.004$ for the Down's syndrome cases).

Figure 6.2

Inhibin-A (●) and free β-hCG (○) levels, expressed as MoMs, in 76 singleton Down's syndrome pregnancies at 10-13 weeks of pregnancy inclusive.



6.4 Discussion and conclusions

The studies in this chapter have explored in some detail the value of inhibin-A as a first trimester marker of Down's syndrome and represent as extension of the small preliminary study related in *Chapter Four*.

Study One reported here related extremely promising data with a median inhibin-A level in the Down's syndrome pregnancies, at 11-13 weeks, almost 2.5x those in the normal controls. This degree of elevation was greater than that found in the second trimester where levels are 1.9x higher than normal (Cuckle *et al* 1996). This was perhaps a little surprising since immunoreactive inhibin can differentiate between normals and Down's syndrome cases in the second trimester (van Lith *et al* 1992, Spencer *et al* 1993, Cuckle *et al* 1994) but not the first (*Chapter Four*, van Lith *et al* 1995) and levels of free inhibin α-subunit, relative to dimeric inhibin, are higher at the earlier gestations (Illingworth *et al* 1996). However, in support of this finding are the subsequent data of Spencer and his colleagues (1996) who observed that inhibin-A levels in Down's syndrome pregnancies were higher in the early weeks of the second trimester, compared to weeks 16-20. Together with the studies described in *Chapter Five*, these data therefore suggested that inhibin-A might be a powerful new marker of Down's syndrome in both the first and second trimesters.

Study Two explored this possibility further and suggested that while inhibin-A did appear to be a potentially useful marker at 13-14 weeks of gestation, the levels at 7-10 weeks of gestation in the Down's syndrome pregnancies were not significantly different from normal. In comparison, levels of intact hCG in the Down's syndrome cases were not significantly different at either 7-10 or 13-14 weeks of pregnancy while free β-hCG levels were significantly elevated at both time points, confirming previous reports (Cuckle *et al* 1988, Macri *et al* 1993). This small study (14 Down's syndrome samples only) therefore confirmed and extended the findings of Study One, suggesting that effective first and second trimester Down's syndrome screening would indeed be possible with inhibin-A, albeit to a lower gestational limit of approximately 11 weeks.

However, the data from Study Three, which involved much larger numbers of samples, were not so encouraging. The median inhibin-A level was only 1.46 MoM in the Down's syndrome samples, significantly lower than the levels observed in Studies One and Two. Given the larger numbers of samples examined in Study Three it is likely that this latter study is more representative of the overall population, an

assumption that would be supported by a recent multicentre study of first trimester markers (Wald *et al* 1996). In that study the median (95% CI) inhibin-A level was 1.19 (1.05-1.35) in 77 samples at 8-14 weeks of gestation, with no trends across those weeks of pregnancy. While the four studies (ie three reported here and that of Wald and colleagues) utilised different formats of the same inhibin-A ELISA, Studies One and Two reported here and the study of Wald and his colleagues used essentially the same format but with different calibrators. It is therefore unlikely that the format of the assay is relevant to the differences between the studies and indeed, as discussed in *Chapter Seven*, there is objective evidence to support this hypothesis, for the second trimester at least.

It would therefore appear that the level of inhibin-A in Down's syndrome pregnancies in the first trimester is not as elevated as first observed in Studies One and two. Nonetheless, the degree of elevation is as high as AFP is low (Brambati *et al* 1986, Sciosca *et al* 1987, Crandall *et al* 1991, Cuckle *et al* 1988, Brock *et al* 1990, Aitken *et al* 1993) and the final value of inhibin-A will depend upon how much it adds to the existing markers. In this regard, the data reported in the three studies here are again not very promising. While inhibin-A and PAPP-A levels are independent, unlike in the second trimester studies reported in this thesis (*Chapter Five*) levels of inhibin-A and free β-hCG are significantly correlated in the first trimester. This was also observed by others (Wald *et al* 1996). Further, Studies Two and Three showed that inhibin-A is not as good a marker of Down's syndrome as free β-hCG, re-affirming the latter as a cornerstone of first trimester serum screening.

A first trimester marker of Down's syndrome so far not discussed is nuchal translucency - a measurement taken, on ultrasound, from the nape of the fetal neck (Nicolaides *et al* 1992). Large scale studies from the same group, in high (Nicolaides *et al* 1994) and subsequently low risk populations (Snijders *et al* 1996), have demonstrated that nuchal translucency (NT) is an excellent marker of Down's syndrome with an overall detection rate as high as 80% for a 5% false positive rate. While there is some concern that other centres have been unable to achieve similarly outstanding results (Bewley *et al* 1995, Scott *et al* 1996) it remains likely that nuchal translucency will find an important role in early pregnancy screening for Down's syndrome.

Importantly, nuchal translucency and serum markers are independent in their assessment of Down's syndrome risk (Noble *et al* 1995), and the addition of free β-hCG to maternal age and nuchal translucency may afford detection rates as high as

85-90% (Noble *et al* 1995) and while the further addition of PAPP-A has not yet been reported, this combination may increase the detection rate yet further for a fixed false positive rate. In such combinations it has been estimated, using modelling, that inhibin-A might add less than 2% to any detection rates (Noble *et al* 1997).

In conclusion, while inhibin-A promises to be a most valuable new marker of Down's syndrome in the second trimester of pregnancy, and it remains elevated in such pregnancies in the first trimester, its is unlikely to find an application as a first trimester serum marker. Rather, the data from all existing studies would suggest that inhibin-A may become useful at approximately 13 weeks of pregnancy, when PAPP-A is losing its power of discernment.

Chapter Seven

Evolution of the inhibin-A ELISA: effects on the detection of Down's syndrome

7.1 Introduction

Chapter Two, Assay Methodology and Validation, (page 18) details the changes that were successively made to the inhibin-A ELISA including the introduction of a pre-assay boiling step, the use of a simplified signal detection reagent and the calibration and introduction of a partially immunopurified standard preparation to replace the recombinant human preparation that had been a gift from Genentech Inc, USA. These modifications improved assay performance, reduced assay costs and allowed widespread commercial distribution of the assay reagents, respectively. However, it was apparent that the use of different assay formats, and the two different inhibin standard preparations within those formats, resulted in considerable variation in the absolute levels of inhibin-A reported, as is evident in the data reported in *Chapters Three, Four and Five.* This has understandably made direct comparisons between different series difficult, an issue of some concern when assessing the value of inhibin-A as a possible prenatal marker of Down's syndrome (Spencer *et al* 1996). Indeed, while no formal comparison was performed it appeared that the earlier studies using the original ELISA format (Wallace *et al* 1995, Wallace *et al* 1996, Aitken *et al* 1996) afforded a better discrimination between Down's syndrome cases and controls than the subsequent reports that used a modified format (Spencer *et al* 1996, Cuckle *et al* 1996, Noble *et al* 1997). While it was likely that this latter observation was simply due to either chance, regression to the mean, or to differences in the sample sets, an effect related to the assay modifications could not be excluded.

In the hope of excluding the latter possibility, that the assay modifications had detrimentally affected the performance of "inhibin-A" as a prenatal marker of Down's syndrome, which if true would represent a significant problem, a simple, quick "experiment" was performed. In this experiment the remaining aliquots from the Down's syndrome samples used in the first large-scale study (reported in *Chapter Five,* page 67 and in Aitken *et al* 1996) were retrieved from frozen storage in Edinburgh and re-assayed using the fully modified assay format. Reflecting the informal nature of this experiment the original controls were not re-assayed but instead the samples used to define the normal range, as detailed in *Chapter Three,* page 35, were used to calculate the median MoM for the Down's syndrome samples. Unfortunately, rather than confirming that the median MoM remained unaffected by the modifications the "new" Down's syndrome MoM was significantly lower than that originally reported (1.43 v 2.28 for the same samples).

Clearly, the approach to the potential format effect problem had not assisted in clarifying the issue at all. Nonetheless, the results, even allowing for the confounding problems of different controls and the extended frozen storage in an extremely small sample volume, when considered with the drift apparent from the successive studies (table 7.4, page 104) mandated a formal comparative study. This chapter details that study and results.

7.2 Material and Methods

7.2.1 Inhibin-A ELISA formats

The original ELISA format and modifications, including the two different standard preparations that have been used, are described in detail in *Chapter Two*. However, for ease of reading the differences in the three formats (I-III) are briefly summarised here.

Format I is the original ELISA method described by Groome and O'Brien (1993) and subsequently validated for human serum (Groome *et al* 1994). Format II is identical to the original method, except that a simplified and cheaper method of detection using a non-amplified alkaline phosphatase substrate, p-nitrophenylphosphate (pNPP), (Kirkegaard and Perry Laboratories, Maryland, USA), was used. This method was validated in-house by Groome and first used in the study described in *Chapter Four* and subsequently reported by Noble and her colleagues (Noble *et al* 1997). Format III involves an additional sample preparation step, performed prior to oxidation, involving heating the sample or standard in a waterbath at 100°C for 3 minutes after mixing with 2% (final w/v) sodium dodecylsulphate (SDS). Detection is afforded by the addition of pNPP as detailed in format II. This format was validated for use in serum and amniotic fluid as described in *Chapter Three* and has been used for inhibin-A measurement in two separate series of Down's syndrome samples (Spencer *et al* 1996, Cuckle *et al* 1996).

The standard preparation used initially was a 32kD recombinant human (rh) inhibin preparation generously supplied by Genentech Inc (CA, USA). This preparation was used in a number of studies, including those reported in *Chapters Four and Five* and those subsequently published (Wallace *et al* 1995, Aitken *et al* 1996, Cuckle *et al* 1995). This preparation has been denoted RH. The second preparation (IP) was developed by Groome, allowing the commercial distribution of the assay. This is partially immunopurified from human follicular fluid and was calibrated by Groome

against the rh inhibin. Six possible permutations of the formats and standards are therefore possible and all six were compared in this study: I-IP, II-IP, III-IP, I-RH, II-RH, III-RH.

7.2.2 Serum samples

Second trimester maternal sera from 41 Down's syndrome pregnancies and 247 control samples, matched for gestation and duration of storage, were identified from records and retrieved. The samples were generously supplied by Dr Kevin Spencer and Dr David Aitken as a collaborative project. None of the Down's syndrome or control samples had been assayed for inhibin-A previously and each sample was assayed blinded to which group it was from. Each sample was assayed by all three formats with both standards consecutively, without further freeze-thawing.

7.2.3 Statistical analyses

Statistical analyses were performed using the software packages Statview 4.1 (Abacus Inc., Berkeley, CA, USA) and SPSS (SPSS Inc, Chicago, IL, USA). Log_{10}(inhibin-A) fits a Gaussian distribution, as previously described in *Chapter Five* and therefore comparisons of absolute levels were made using log_{10} levels. Because inhibin-A levels vary significantly with gestation, as described in *Chapter Three*, regressed medians were calculated for each week of gestation, as previously described in *Chapter Three* (page 37) and levels in the Down's syndrome samples expressed as multiples of these normal medians (MoMs). MoMs obtained with different formats were compared by Wilcoxon rank sums for paired tests. Distribution parameters were calculated for inhibin-A for each of the formats by Dr Jenny Crossley (see *Acknowledgments*, page viii). The mean was taken as log_{10}median and the standard deviation as the difference between the 10th and 90th centiles in logs, divided by 2.56 (Cuckle *et al* 1987). Comparative analyses were recorded as significant when $p<0.05$.

7.3 Results

An assay problem was encountered for the first batch of samples assayed with format I (both standards). There was not sufficient sample volume to re-assay these samples and so data are available for this format for fewer samples (both controls and Down's syndrome) than the other two formats. Further, there was insufficient volume in another Down's syndrome sample to perform all three assay formats and so there are no data available on this sample assayed by format III.

7.3.1 Chromosomally normal control samples.

Median inhibin-A levels in the control samples differed significantly between the three formats. In particular, the levels obtained by format III-RH were approximately double those of the other formats (Table 7.1).

Table 7.1.
Regressed median inhibin-A levels in 247 chromosomally normal pregnancies at 15-20 weeks gestation.

Gestation	**Format I**			**Format II**			**Format III**		
(weeks)	n	IP	RH	n	IP	RH	n	IP	RH
15	49	134.7	146.5	96	120.4	139.3	96	134.1	357.9
16	33	133.0	146.0	64	111.8	129.6	64	129.0	341.9
17	49	145.6	162.1	65	113.6	132.6	65	127.4	339.0
18	7	172.4	194.6	12	125.8	148.5	12	129.2	349.0
19	3	213.4	243.5	5	148.8	177.1	5	134.4	371.9
20				5	181.6	218.6	5	143.0	407.8

The parameters of inhibin-A distribution in the control samples also varied between formats with format III affording the narrowest width of distribution, irrespective of the standard used (Table 7.2, page 99).

7.3.2 Down's syndrome samples

As with the control samples, the parameters of inhibin-A distribution in the Down's syndrome samples varied between formats with format III affording the narrowest width of distribution, irrespective of the standard used (Table 7.2, page 99). The median inhibin-A level in the Down's syndrome samples, expressed as MoMs, was significantly higher in formats II and III, when derived with the RH standard than when derived with the IP standard (table 7.2, $p<0.0001$ for both). There were no significant differences between standards for format I ($p=0.09$). For a given standard preparation no particular format afforded persistently higher Down's syndrome MoMs than the others although, for the RH standard, the median MoM was significantly

higher when derived with format III than with format II (p=0.02) and for the IP standard, higher with format I than with format II (p=0.01). There were no other significant differences. Consistent with these trends, the distance from the control mean, a measurement of separation, of Down's sample mean was greatest with format III than the others.

Table 7.2.
Parameters of distribution of inhibin-A levels (MoM) in serum from 247 chromosomally normal and 41 Down's syndrome pregnancies. Mean and SD are of log gaussian distributions. (Upper - controls, *Lower - Down's syndrome)*

	n	Median	Mean	SD	Distance from control mean (SD)
I - IP	141	1.01	0.003	0.238	-
	19	*1.73*	*0.239*	*0.392*	*0.99*
II - IP	247	1.02	0.006	0.293	-
	41	*1.54*	*0.188*	*0.408*	*0.62*
III - IP	247	1.00	0.002	0.182	-
	40	*1.72*	*0.236*	*0.203*	*1.29*
I - RH	141	1.02	0.008	0.377	-
	19	*1.85*	*0.267*	*0.778*	*0.69*
II - RH	247	1.01	0.004	0.425	-
	41	*1.83*	*0.262*	*0.629*	*0.61*
III-RH	247	1.01	0.002	0.2209	-
	40	*1.88*	*0.275*	*0.2496*	*1.23*

Table 7.3 (page 99) displays the correlation coefficients between Log_{10}(inhibin-A) levels in the control and Down's syndrome samples obtained by the different formats.

The highest coefficients of correlation are between levels derived from the two different standard preparations within a given assay format. Further, there is generally a high level of correlation between the levels obtained with assay formats I and II (irrespective of standard preparation), whereas the inhibin values derived with assay format III correlate less well with those from the other two formats.

Table 7.3.
Correlation coefficients (r) between Log_{10}(inhibin-A) in Down's syndrome and control samples for the three assay formats (I-III) and two standard preparations (IP, RH). (Upper - controls, *Lower - Down's syndrome)*

	II - IP	**III - IP**	**I - RH**	**II - RH**	**III - RH**
I - IP	0.86 *0.92*	0.61 *0.45*	0.94 *0.80*	0.79 *0.87*	0.62 *0.46*
II - IP	-	0.61 *0.52*	0.74 *0.92*	0.98 *0.98*	0.61 *0.52*
III - IP	-	-	0.57 *0.42*	0.65 *0.46*	0.99 *1.0*
I - RH	-	-	-	0.78 *0.91*	0.57 *0.43*
II - RH	-	-	-	-	0.67 *0.56*

7.4 Discussion and conclusions

This study has afforded for the first time, formal comparative data from the different inhibin-A ELISA formats. As was apparent from the previous studies related in *Chapters Three, Four* and *Five* and reported in the literature (Wallace *et al* 1995, Cuckle *et al* 1995, Aitken *et al* 1996, Wallace *et al* 1996, Spencer *et al* 1996, Cuckle *et al* 1996, Noble *et al* 1997), the data reported here confirm that there are significant differences in absolute levels of inhibin reported depending on the ELISA format and standard preparation used (table 7.1). However, rather than simply confirming these differences this study also affords some explanation for them.

Because the three ELISA formats remain highly specific for inhibin-A and because the IP standard was calibrated against the RH standard, differences between the formats

cannot have arisen from the detection of other inhibin moieties and must be due to variations in the efficacy of the formats to detect inhibin-A. Biological fluids contain a number of different molecular weight forms of inhibins (Schneyer *et al* 1990, Robertson *et al* 1995, Robertson *et al* 1996) and it is now apparent that the higher molecular weight inhibin-A forms, as well as probably being biologically inactive (Mason *et al* 1996), are less immunoreactive in the inhibin-A ELISA used here than 32kDa inhibin-A (Good *et al* 1995, Priddle *et al* 1995). However, the immunoreactivity of the larger forms can be increased approximately two-fold by pre-assay boiling (Mason *et al* 1996), an effect that cannot be secondary to degradation, because the large inhibin forms used were engineered to be non-cleavable. The increased levels observed after boiling therefore, presumably arise through improved epitope exposure, perhaps through protein unfolding. The extent of this effect after boiling is very similar to the approximately two-fold increase in inhibin-A levels observed in this study with format III compared to the other two formats. It is therefore proposed that the differences evident here relate to changes in epitope exposure following the SDS boiling step.

That these differences are only evident with III-RH, and not III-IP, is explained by the composition of the two standard preparations. The RH standard, a recombinant preparation, contains only 32kDa rh inhibin-A and thus boiling in SDS would not be expected to change the already optimum antibody-epitope binding. However, boiling would improve antibody binding to the epitopes of the larger molecular weight inhibin-A forms present in the unknown samples. The net effect therefore is a relative increase in the inhibin-A measured against the recombinant standards. The IP standard however was derived from human follicular fluid which contains many different molecular weight inhibins (Robertson *et al* 1995). Thus, when the IP standard is employed to derive unknown values the effect of boiling is exerted on both the standard and the sample resulting in minimal change in absolute inhibin levels. The precise net effect is dependent on the relative concentrations of the different forms in the standard (derived from follicular fluid) and sample (serum).

In *Chapter Three*, page 52, the ontogeny of inhibin-A in maternal serum in early pregnancy is described in detail. The findings of Muttakrishna and her colleagues (1995) are discussed in that chapter, relating how those authors used chromatography to demonstrate that in pregnancy only 32kD inhibin-A circulated. However, if this were true then the effects described above could not arise. The presence of only 32kD inhibin in serum would mean that when assayed against the RH standard, the boiling pretreatment would confer no changes (ie the inhibins in the standards and unknowns

are identical). In contrast, when assayed against the IP standard inhibin-A levels would fall (reflecting improved antibody affinity for the unfolded epitopes in the standards compared to the unaltered affinity for the already optimum epitopes in the unknowns). This is exactly the opposite of what is observed. Thus, the data reported here provide indirect evidence that different molecular sizes of inhibin-A do circulate in early pregnancy, supporting the preliminary observations made with two different immunoreactive inhibin assays, as detailed in *Chapter Four*, page 60, and the chromatographic data of Khalil and his colleagues (1995). It is likely therefore that in the study of Muttakrishna and colleagues (1995) problems with sample collection and / or storage resulted in *in vitro* degradation of the inhibin prior to chromatography.

However, the poor correlations between format III derived inhibin-A levels and format I or II derived levels is probably not due to the differential detection of various inhibin-A forms. If this was so then a poor correlation would be expected between the levels derived with III-IP and those from III-RH. This was not observed. It is therefore proposed that these differences relate to the release of erythrocyte catalase in the venous samples. Catalase would remove the hydrogen peroxide added during pretreatment and thus prevent modification of the inhibin β-subunit and so impair detection. This effect is evident in some samples, presumably depending on storage/separation history, when assayed by formats I and II, but would be overcome by the SDS boiling pretreatment in format III, probably through the denaturing of the catalase. While catalase was not measured in the samples to confirm this directly, indirect evidence is offered by the stability data related in *Chapter Two*, page 26. These data showed that inhibin-A levels fell when samples were stored as whole blood but not as serum, and that when the samples were boiled prior to assay the decline was prevented. Thus, the changes observed with format II (no boiling) could not have been due to dissociation of the inhibin dimer, because levels were stable with format III (SDS boiling), but rather to an effect related to storage as whole blood, but not serum. Importantly, a quenched inhibin-A signal was evident in the stability study samples even without obvious haemolysis. These data therefore suggest that while the apparent inhibin-A levels is stable in clinical samples, this is only so when using format III. In the context of possible prenatal screening programmes, when samples might travel for some distance and time prior to assay, this is an important observation. Indeed, there have been important concerns about similar changes in fβ-hCG levels after sampling but prior to assay, concerns that may have limited the uptake of this analyte (Sancken and Bahner 1995, Kardana and Cole 1997)

Further support to the catalase hypothesis is given by finding that the correlation coefficient between results from formats II-IP and III-IP can be increased by recalculating using only those samples with an inhibin-A level of at least 70pg/mL in formats I and II (r=0.76 v r=0.61). 70pg/mL approximates to the 10th centile (see *Chapter Three*, page 40) and so excludes many samples that may have an inhibin-A level artefactually lowered by catalase. Similarly, the tighter distribution of results with format III compared to the other two formats is probably an effect of normalising the levels in some samples with a quenched signal.

The high degree of correlation between the results derived with formats I and II suggests that, while there may be small differences in absolute levels detected, the method of detection of inhibin-A in the ELISA is not of critical importance, so long as expected levels are above the sensitivity of the method employed. Therefore, given the considerable cost savings afforded by the simple substrate detection method compared to the amplification kits the former methodology appears preferable.

Lastly, in this study, the boiling pretreatment was associated with an apparent modest improvement in the discrimination between Down's syndrome samples and controls (table 7.2, page 97), the opposite of the concerns outlined in the *Introduction* to this chapter that initiated this study. To aid interpretation of these data in the context of the existing literature table 7.4 summarises the previous second trimester studies (some of which have been described in earlier chapters), detailing the format used in each.

Interestingly, the most recent, and largest, of these studies utilised format III-IP (Spencer *et al* 1996) and reported results less promising than the earlier studies using format I (Wallace *et al* 1996, Aitken *et al* 1996), as outlined in *Chapter Five*. Considering the biology underlying the differences between the formats and the current understanding of inhibin-A in normal and Down's syndrome pregnancy (Wallace and Healy 1996, Wallace *et al* 1997) it is unlikely that the assay format will impact significantly upon detection rates (table 7.4). In support of this, two sample sets reported from the same centre and analysed as work related to this thesis, one analysed with format I (Cuckle *et al* 1995) and one with format III (Cuckle *et al* 1996), showed no significant differences in terms of median MoMs.

Table 7.4.

Inhibin-A ELISA method used and median affected inhibin-A MoM reported in previous second trimester studies.

Author	ELISA method	Median Inhibin-A MoM in Affected Pregnancies
Cuckle et al 1995	I-RH	1.64
Wallace et al 1996	I-RH	2.6
Aitken et al 1996	I-RH	2.24
Wald et al 1996	I-RH*	1.79
Spencer et al 1996	III-IP	1.77
Cuckle et al 1996	III-IP	1.58

* the recombinant standard used by Wald *et al* differs from the rh-inhibin-A preparation (RH) referred to in this chapter.

In conclusion, this is the first and only formal comparison of three different formats of the inhibin-A ELISA. The data reported here demonstrate that while the absolute levels of inhibin-A reported depend on the assay format used, the discrimination of Down's syndrome cases from controls would not appear to be significantly affected. Nonetheless, the boiling pretreatment affords significant improvements in assay performance, particularly for samples stored as whole blood, and on this basis format III would appear to be the most useful clinically. The introduction of an internationally agreed inhibin-A standard will prevent other differences between laboratories but until then a given institution should plan to use the same standard preparation for their long term studies.

Chapter Eight

Inhibins in Amniotic Fluid in Down's Syndrome Pregnancy

8.1 Introduction

As described in *Chapter Three*, amniotic fluid contains significant amounts of both inhibin dimers and inhibin precursor fragments, probably including free inhibin α-subunits. Further, as discussed in that chapter and supported by indirect evidence, it is likely that the source of these proteins in amniotic fluid is the chorionic trophoblast, rather than the placenta. However, while it is also clear that, compared to normal pregnancy, maternal serum levels of inhibin-A are elevated in Down's syndrome pregnancies, in both the first (see *Chapter Five*; Wallace *et al* 1995, Noble *et al* 1997) and second (see *Chapter Six*; *Cuckle et al* 1995, Wallace *et al* 1996, Aitken *et al* 1996, Spencer *et al* 1996, Cuckle *et al* 1996, Wald *et al* 1996) trimesters inhibin-A levels in amniotic fluid in Down's syndrome pregnancy remains unexplored. If the production and secretion of inhibins from the chorion and placenta are differentially regulated, as suggested in *Chapter Three*, then it is conceivable that AF inhibin levels in Down's syndrome may be normal. Therefore, to further explore the biology of inhibin secretion in pregnancy AF levels of inhibin-A, inhibin-B and pro-αC containing inhibins in chromosomally normal and Down's syndrome pregnancies have been examined.

8.2 Materials and Methods

All samples for this study were generously supplied by Dr David Aitken and Dr Jenny Crossley, Duncan Guthrie Institute, Glasgow (see *Acknowledgments*, page viii)

8.2.1 Control samples

One hundred and sixty-one chromosomally normal control pregnancies on which an amniocentesis had been performed were identified from records and an aliquot of fluid retrieved from storage at -20°C. Sixty-eight (42.2%) of these amniocenteses had been performed for maternal age, 82 (50.9%) for a positive MS screening result, 1 (0.6%) for an isolated elevated MS AFP and 10 (6.2%) for other miscellaneous reasons (eg past history, maternal anxiety). Sample volume was sufficient in 150 of these control aliquots to allow the measurement of inhibin-A, inhibin-B and pro-αC inhibins, whereas in the remaining 11 samples only inhibin-A was measured.

8.2.2 Down's syndrome samples

Similarly, 51 Down's syndrome pregnancies, from which AF had been collected prospectively, were identified from records and an aliquot of fluid retrieved from storage. Of these 51 pregnancies, 11 (21.6%) had an amniocentesis performed because of maternal age and 40 (78.4%) because of an increased risk by AFP/age or AFP/hCG/age screening. In six of the 51 aliquots, the sample volume was insufficient for all three of the assays and only inhibin-A was measured in these. In the remaining 45, inhibin-A, inhibin-B and pro-αC inhibin levels are reported.

8.2.3 Inhibin assays

The three inhibin assays have been described and validated for amniotic fluid previously (*Chapter Three*). Of the inhibin-A assay formats, format III was used in this study. All samples were assayed blinded to whether the sample was from a Down's syndrome or a normal pregnancy

8.2.4 Statistical analyses

Statistical analyses were performed using Statview 4.1 (Abacus Inc, Berkeley, CA, USA) and SPSS for Windows (SPSS Inc, Chicago, IL, USA). AF inhibin-A and inhibin-B levels increase significantly and linearly across the gestational window 14 to 20 weeks (*Chapter Three*). The levels of these two proteins in both the Down's syndrome and control pregnancies were therefore expressed as multiples of the normal median (MoM), using the regressed median of the controls at the appropriate gestation. The use of regressed medians corrects for the artefactual variation that arises secondary to the small sample size and so affords more accurate MoM estimates for both the control and Down's syndrome samples than using non-regressed medians. The regressed medians were calculated, by Dr Jenny Crossley, using the weighted regression equations:

$$\text{Inhibin-A median} = e^{(8.0649 - 35.045/\text{gestation})}$$

$$\text{Inhibin-B median} = e^{(8.4178 - 41.880/\text{gestation})}$$

Weighted regression of the pro-αC inhibin levels however did not generate an equation that fitted the data well. Pro-αC inhibin MoMs have therefore been calculated using raw, rather than regressed, medians. An analysis of both approaches showed no significant differences in terms of the resulting comparison between controls and cases (data not shown).

8.3 Results

8.3.1 Control samples

78 (48.4%) of the 161 control AF samples were from pregnancies with a female fetus, 82 (50.9%) were from a pregnancy with a male fetus and 1 (0.6%) was unknown. Of the Down's syndrome samples, 18 (37.3%) and 32 (62.7%) were from pregnancies with a female and male fetus respectively. There were no significant differences in inhibin-A, inhibin-B or pro-αC inhibin levels between the sexes for either controls or Down's syndrome cases (data not shown) and so the data were subsequently analysed by group, combining data for both sexes therein. Table 8.1 (page 108) displays the number of control samples at each gestation and the median, 10th and 90th percentiles for inhibin-A, inhibin-B and pro-αC inhibins in these samples.

Inhibin-A was weakly but significantly associated with inhibin-B ($r=0.44$, $p=0.0001$) and pro-αC inhibins ($r=0.34$, $p=0.0001$) and inhibin-B was weakly but significantly associated with pro-αC inhibins ($r=0.24$, $p=0.005$).

8.3.2 Down's syndrome samples

Table 8.1 (page 108) displays the number of Down's syndrome samples at each gestation and the median, 10th and 90th percentiles for inhibin-A, inhibin-B and pro-αC inhibins in these samples. Expressed as MoMs the median (95% CI) level for the three inhibins was 0.77 (0.68-0.89) for inhibin-A, 0.90 (0.52-1.57) for inhibin-B and 0.79 (0.63-1.13) for pro-αC inhibins. The level of inhibin-A in AF from the Down's syndrome pregnancies was significantly lower than in the controls ($p<0.0002$ Mann-Whitney U test), whereas the level of neither of the other inhibins was significantly lower than normal ($p=0.35$ and $p=0.49$, respectively; Mann-Whitney U test).

In contrast to the controls, in the Down's syndrome samples, there was no significant association between inhibin-A and inhibin-B ($r=0.19$, $p=0.21$), whereas pro-αC inhibins were significantly associated with both inhibin-A ($r=0.36$, $p=0.0002$) and inhibin-B ($r=0.41$, $p=0.03$).

Table 8.1

Levels (pg/mL) of inhibin-A, inhibin-B and pro-aC containing inhibin in AF from 161 chromosomally normal and 51 Down's syndrome pregnancies. n= number of samples.

		16 weeks gestation			17 weeks gestation			18 weeks gestation			19 weeks gestation		
		n	median	10th-90th percentiles	n	median	10th-90th percentiles	n	median	10th-90th percentiles	n	median	10th-90th percentiles
Inhibin-A	control	45	339.6	175.2-659.1	46	485.7	221.5-829.7	46	362.2	138.4-893.0	24	592.9	256.4-1027.3
	Down's syndrome	*11*	*158.9*	*106.4-387.6*	*16*	*314.7*	*99.2-558.1*	*21*	*349.3*	*170.6-560.8*	*3*	*430.1*	
Inhibin-B	control	43	310.0	80.8-1112.5	40	426.2	144.5-1031.4	40	449.9	182.0-2505.1	24	459.5	193.7-1386.8
	Down's syndrome	*11*	*139.3*	*54.0-1195.8*	*11*	*352.1*	*139.0-1462.4*	*19*	*565.1*	*196.1-985.6*	*3*	*1030*	
Pro-αC inhibins	control	41	541.8	206.9-1322.8	38	773.6	246.9-1625.2	43	552.0	185.4-1212.9	24	1391.8	433.3-2652.6
	Down's syndrome	*10*	*429.3*	*216.7-1266.9*	*11*	*489.4*	*186.3-941.6*	*20*	*571.0*	*199.0-1581.0*	*3*	*1267.4*	

8.4 Discussion and conclusions

This is the first study to examine AF levels of inhibin-A, inhibin-B and pro-αC inhibins in Down's syndrome and normal pregnancy and the results may shed some light on the control of inhibin secretion in pregnancy.

While the ontogeny of inhibin and hCG in early pregnancy are similar (Tovanabutra *et al* 1993, Illingworth *et al* 1996), possibly reflecting reciprocal *in vitro* relationships (Petraglia *et al* 1987), and maternal serum levels of both are elevated in Down's syndrome (see *Chapters Five* and *Six*, van Lith *et al* 1992, Spencer *et al* 1993, Bogart *et al* 1987, Chard and Iles 1994), which for hCG at least is secondary to increased placental production (Eldar-Geva *et al* 1995), there are important differences between the secretion of hCG and inhibin in both normal and Down's syndrome pregnancies. In normal pregnancy the intact hCG concentration is much lower in AF than in MS (Kletsky *et al* 1985) while inhibin-A concentrations in AF are significantly higher than in MS (*Chapter Three*, page 44). Further, in Down's syndrome pregnancies while both intact hCG and free βhCG levels in AF are higher than in AF from normal pregnancies (Cuckle *et al* 1991, Spencer *et al* 1997), comparable with the differences reported in serum (Bogart *et al* 1987, Chard and Iles 1994), this study has shown that AF levels of inhibin-A in Down's syndrome pregnancies are significantly lower than in normal pregnancy AF, the reverse of what is observed in MS for this protein. Of course, since inhibin-B is undetectable in maternal serum at this gestation (*Chapter Three*, page 42), no comparisons between AF and MS can be made. There are currently no reports of MS levels of pro-αC in Down's syndrome pregnancies although it has been suggested, based on indirect evidence, that these will not be significantly different from normal (Wallace and Healy 1996).

The lower levels of AF inhibin-A observed in Down's syndrome compared to normal may relate to changes in either the route of secretion of inhibin-A by the placenta, in preference for MS, or in the rate of clearance of inhibin-A from the amniotic cavity. While MS levels of several proteins and steroids are deranged in Down's syndrome, the AF levels for each of these are similarly deranged (Cuckle *et al* 1985, Cuckle *et al* 1991, Spencer *et al* 1997), indicative of neither an altered route of secretion nor selective changes in clearance from AF. This explanation for the inhibin-A observations is therefore unlikely. It is conceivable however, that dimeric inhibin-A, but not inhibin-B, in AF, but not MS, may dissociate into separate subunits more rapidly in Down's syndrome relative to normal pregnancy. Levels of free βhCG and free αhCG subunits are much

higher in AF than in MS while intact hCG levels are lower (Ozturk *et al* 1988). The increased concentration of free hCG subunits in AF compared to MS could be accounted for by an increased breakdown of intact hCG in AF but it is thought more likely that the chorionic trophoblast preferentially secretes free subunits into AF and the placental trophoblast secretes intact hCG into MS (Ozturk *et al* 1988). In the first trimester of pregnancy the major source of hCG in extra-embryonic coelom, and thereafter AF, is probably chorionic, as opposed to placental, trophoblast (Iles *et al* 1992, Chard *et al* 1995). It is therefore possible that in the second trimester the differences between MS and AF in intact and free subunit hCG levels may represent different sources.

Thus, the source of AF inhibin-A may be different from that of MS, as previously suggested (*Chapter Three*). If this were so then the data related here would indicate that the abnormality in Down's syndrome underlying the elevated MS inhibin-A levels would have to be differentially expressed in the placental and chorionic trophoblast. This would be quite unlike hCG (either intact or free βhCG).

The weak but significant associations between AF levels of inhibin-A, inhibin-B and pro-αC inhibins in the control pregnancies is largely in keeping with the previous studies of extra-embryonic coelomic fluid and AF in the first and second trimesters of chromosomally normal pregnancies (*Chapter Three*). In the Down's syndrome pregnancies however, inhibin-A and inhibin-B were not associated. While this may simply be due to the smaller numbers of cases compared to controls, this observation, together with the observation that the levels of inhibin-B and pro-aC inhibins were not significantly lower than normal, may indicate that the abnormality underlying the decreased inhibin-A levels is expressed specifically through the production or secretion of the inhibin β_A subunit, rather than the other two subunits.

In conclusion, this study has demonstrated that the amniotic fluid inhibin-A, but not inhibin-B or pro-αC, concentration in Down's syndrome is significantly lower than in normal pregnancy. The precise explanation for these unexpected findings currently remains obscure but the data are consistent with more than one significant source of inhibins in mid-pregnancy and with differential control of these sources. Quantitative assessment of the inhibin subunit messenger ribonucleic acids (mRNAs) and proteins in fetal membranes in normal and Down's syndrome pregnancies may contribute to the understanding of the changes in inhibin secretion in trisomy 21 pregnancies.

Chapter Nine

Diabetes Mellitus and Maternal Serum Inhibin-A Levels

9.1 Introduction

A number of maternal factors are known to affect the serum level of different Down's syndrome markers and so potentially affect the predicted risk of having a Down's syndrome fetus including race (Muller and Boué 1990, Simpson *et al* 1990, Kulch *et al* 1993, Bogart *et al* 1991), cigarette use (Bernstein *et al* 1989, Cuckle *et al* 1990, Bartels *et al* 1993), early pregnancy bleeding (Salem *et al* 1984) and insulin dependant diabetes mellitus (IDDM) (Wald *et al* 1979, Milunsky *et al* 1982, Reece *et al* 1987, Wald *et al* 1992, Palomaki *et al* 1994). In particular, it has been reported that early second trimester serum levels of AFP and hCG in women with IDDM are lower than in non-diabetic women (Wald *et al* 1979, Milunsky *et al* 1982, Reece *et al* 1987, Wald *et al* 1992, Palomaki *et al* 1994). On average, AFP levels have been found to be 70% of those in non-diabetics which, while a small effect, may artefactually increase the predicted risk of a given pregnancy. In contrast, the lower hCG level observed in women with IDDM, on average levels are 95% of those in non-diabetics, might be expected to decrease estimated risks.

Therefore, in view of the potential usefulness of inhibin-A as a prenatal marker of Down's syndrome (see *Chapter Five*) it was decided to compare early second trimester inhibin-A levels in the serum of pregnant women with IDDM with those in non-diabetic women.

9.2 Material and Methods

9.2.1 Serum samples

Maternal serum samples were collected prospectively as part of routine the West of Scotland Down's syndrome and neural tube defects prenatal screening programme. Each sample had been centrifuged and the serum separated within three days of collection and stored at -20°C. 169 women with pre-existing IDDM and a chromosomally normal, singleton pregnancy were identified from records and their stored serum retrieved for analysis. These are a subset of 261 IDDM samples on which hCG and AFP have been reported previously (Crossley *et al* 1996). Similarly, sera from 432 non-diabetic women with a chromosomally normal singleton pregnancy, matched for gestation and duration of storage with the 169 IDDM samples, were identified and retrieved. These represent a subset of the normal pregnancy samples reported in *Chapter Three*, page 35.

Gestation, in completed weeks of pregnancy, was calculated from certain menstrual dates or by an early pregnancy ultrasound scan.

9.2.2 Inhibin-A assay

Inhibin-A was measured using format III of the inhibin-A ELISA, as detailed in *Chapters Two* and *Seven.*

9.2.3 Statistical analyses

Statistical analyses were performed using Statview 4.1 (Abacus Inc, Berkeley, CA, USA) and SPSS for Windows (SPSS Inc., Chicago, IL, USA). Inhibin-A levels were converted to multiples of the normal median for gestation, in completed weeks, using regressed medians as previously described and validated in *Chapter Three.* As detailed in *Chapter Five* inhibin-A MoM is log gaussian distributed. Therefore, to allow comparisons, where appropriate, the mean and SD of $\log_{10}$(MoM) were calculated. The mean was estimated as $\log_{10}$ of the median and the SD as ($\log_{10}$(90th centile) - $\log_{10}$(10th centile))/2.56 (Cuckle *et al* 1987). The correlation between inhibin-A and hCG levels was examined using log(MoM) for each analyte.

To correct inhibin-A levels for maternal weight weighted log-linear regression of the median inhibin-A MoM against median weight, in 10kg weight groups, was performed. This generated the equation:

$$\text{Weight corrected inhibin-A MoM} = \text{inhibin-A MoM} / 10^{(0.2488-0.0040 \times \text{weight})}.$$

The regression calculations, both for the median inhibin-A values and for the maternal weight corrections were kindly performed by Dr Jenny Crossley (see *Acknowledgments*, page viii).

9.3 Results

Table 9.1 (page 115) shows the number of samples, the 10th, 50th and 90th percentiles for inhibin-A in the 432 non-IDDM and 169 IDDM pregnancies by completed week of gestation. Levels of inhibin-A fell from 15 weeks to 17 weeks rising again to 20 weeks.

Table 9.2 (page 115) shows the median, mean and standard deviation of the inhibin-A, expressed as MoMs, in both the control and IDDM pregnancies. There were no

significant differences between the two groups when no adjustments were made for maternal weight ($p>0.05$, Student's t-test).

Table 9.1.
Median (10th-90th percentile) maternal serum inhibin-A levels in 432 non-diabetic women and 169 women with IDDM.

Gestation (weeks)	**Controls**		**IDDM**	
	No. of samples	Inhibin-A (pg/mL)	No. of samples	Inhibin-A (pg/mL)
15	75	123.5 (73.2 - 261.2)	58	126.9 (59.8-252.3)
16	75	104.3 (54.2-189.4)	69	115.7 (47.4-227.3)
17	75	100.5 (59.5-200.3)	25	102.4 (51.1-189.6)
18	75	122.4 (67.3-291.2)	12	115.7 (81.9-193.1)
19	75	118.7 (61.2-277.4)	4	139.6
20	57	144.8 (74.1-327.2)	1	487.8

Table 9.2.
Medians, means and SDs of maternal serum inhibin-A in 432 normal and 169 IDDM pregnancies.

	Controls			**IDDM**			
	Median	Mean	SD	Median	Mean	SD	significance
Inhibin-A (MoM)	1.01	0.003	0.240	1.05	0.022	0.250	ns
Inhibin-A (MoM) (weight corrected)	1.02	0.010	0.219	1.17	0.068	0.241	$p<0.01$

Maternal weight at sampling was available for 391 (91%) of the control samples and 151 (89%) of the IDDM samples. The median maternal weights of these two groups were 63kg and 68kg respectively. Table 9.3 shows the median inhibin-A MoM for the control samples, divided into 10kg weight bands, showing a decline in median MoM with increasing weight. Table 9.2 shows the weight corrected median, mean and SD inhibin-A MoM. When weight-adjusted, the inhibin-A level in the IDDM group was significantly higher than that in the controls ($p<0.01$, Student's t-test).

Table 9.3.
Distribution, by weight, of 391 control pregnancies and the relationship between weight and inhibin-A levels.

Weight Group (kg)	**n**	**Median weight (kg)**	**Median inhibin-A (MoM)**
<50	25	46.5	1.013
50 - 59.99	115	55.5	1.125
60 - 69.99	150	64.0	1.005
70 - 79.99	62	73.0	0.822
≥ 80	39	86.5	0.852

Weight corrected inhibin-A and hCG levels were significantly correlated ($r=0.32$, $p<0.001$ and $r=0.22$, $p<0.001$ for the IDDM women and the controls respectively).

9.3 Discussion and conclusions

As detailed in the Introduction to this chapter it has been shown previously that, compared to non-diabetic women, maternal serum levels of AFP and hCG may be significantly lower in pregnancies complicated by IDDM (Wald *et al* 1979, Milunsky *et al* 1982, Reece *et al* 1987, Wald *et al* 1992, Palomaki *et al* 1994). While theoretically such IDDM-related changes could alter risk estimations, and thereby impair the performance of screening programmes, recently Crossley and colleagues (1996)

reported that correction of the serum AFP level for maternal weight, which was on average greater in the diabetic women, normalised the AFP levels in those women. Further, the same authors demonstrated that in actual practice the effects of IDDM on marker levels did not result in any significant alteration in screening follow-up rates amongst diabetic women, suggesting that no formal correction of the minor effects was required. The data reported here show that the serum inhibin-A level is not significantly different between women with and without IDDM, when no adjustments are made for maternal weight. However, the women with IDDM were on average 5kg heavier than the non-diabetic women, as has been reported previously (Palomaki *et al* 1994, Crossley *et al* 1996), and the inhibin-A level was inversely and significantly related to maternal weight, as is hCG (Suchy and Yeager 1990, Bogart *et al* 1991, Bartels *et al* 1993). Correcting the inhibin-A level for this weight effect led to a significantly higher level of inhibin-A in the IDDM women. This observation is in direct contrast to the data recently reported by Wald and his colleagues (1996) who reported that in diabetics the median inhibin-A level was significantly lower (0.88 MoM). When corrected for maternal weight, this effect was lessened and became non-significant (0.91 MoM) (Wald *et al* 1996), but remained reduced rather than increased. At present, it is difficult to explain these apparent contradictory results and considering the *in vitro* and *in vivo* relationships between inhibin and hCG and the relatively lower hCG levels in women with IDDM (Wald *et al* 1992, Palomaki *et al* 1994, Crossley *et al* 1996) it might appear that the data of Wald and his colleagues are the more plausible. However, it is interesting that while Palomaki and his colleagues (1994) reported lower hCG levels overall for the diabetic pregnancies, they found that levels were actually higher (1.18 MoMs) in women who had "early-onset" diabetes, diabetes onset prior to 19 years of age. Further, these women had a mean weight very similar to the non-diabetic population (148 lbs v 151 lbs, respectively) whereas the "late-onset" diabetics were significantly heavier (190 lbs). It is possible therefore that differences in the duration of diabetes may exert subtle endocrine effects and in this regard a significant association between maternal serum AFP and glycosolated haemoglobin, a measure glycaemic control (Leslie *et al* 1978), has been reported (Reece *et al* 1987).

An obvious explanation for the elevated inhibin-A might be changes in circulating insulin and/or insulin-like growth factor (IGF) levels in the diabetic women. However, this is an unlikely explanation for a number of reasons. Firstly, circulating IGF-I levels are lower rather than higher in individuals with IDDM (Tan and Baxter 1986). Furthermore, although fetal serum IGF-I and IGF-II levels are increased in diabetic

pregnancies (Delmis *et al* 1992, Verhaeghe *et al* 1993), in vitro studies have failed to show any effect of insulin, IGF-I or IGF-II on trophoblast secretion of immunoreactive inhibin (Qu and Thomas 1995).

Hyperglycaemia may be the mechanism underlying the altered inhibin-A levels, as has been suggested for AFP (Reece *et al* 1987), and in this regard it is interesting that Petraglia and colleagues observed that maternal serum levels of activin-A, a protein of the same family as inhibin-A (Ying 1988) which is secreted by the placenta (de Kretser *et al* 1994, Qu and Thomas 1995, Knight *et al* 1996), are elevated approximately five fold in the third trimester in women with gestational diabetes (Petraglia *et al* 1995). Further, the activin-A levels normalised when insulin treatment was commenced and euglycaemia was achieved. Activin-A is a homodimer of the β_A subunit, (β_A-β_A), and it is possible that the diabetes-associated elevations of activin-A and inhibin-A are mediated entirely through the regulation of the β_A-subunit. While this is entirely speculative at present it is afforded indirect support by the suggestion that the decreased AF inhibin-A levels observed in Down's syndrome pregnancies may reflect specific changes in inhibin β_A-subunit secretion (*Chapter Eight*).

In vitro, hCG stimulates inhibin secretion and inhibin suppress hCG secretion from dispersed trophoblast cell cultures (Petraglia *et al* 1987, Mersol-Barg *et al* 1990). Therefore, the data presented here may be the first *in vivo* evidence, albeit indirect, to support an hCG suppressive role for inhibin. Arguing against this is the direct rather than inverse relationship observed between hCG and inhibin, which was observed in the studies reported in *Chapter Five* . Further, activin stimulates hCG secretion *in vitro* (Petraglia *et al* 1995) and one would predict from these *in vitro* data that the changes in activin-A levels that might be expected in the diabetic women would more than compensate for the effects of the minimal changes in inhibin-A that were observed.

The control dataset analysed in this study also confirms the observation, made in *Chapters Three* and *Five* that the absolute change in inhibin-A levels between 15 and 20 gestation, the window for second trimester aneuploidy screening, are small. This is particularly true relative to established markers of Down's syndrome. The decline in inhibin-A from 15 to 17 weeks was only 19% and the subsequent increase to 20 weeks 44%, whereas the levels of hCG and AFP approximately half and double, respectively, over the same interval (Westergaard *et al* 1985). This consistent finding suggests that inhibin-A would be less affected by inaccurate gestational dating than is the case for other markers, a most beneficial property for a potential new marker of Down's syndrome.

Thus, it has been demonstrated that, at 15-20 weeks gestation, maternal serum inhibin-A levels in women with insulin-dependent diabetes mellitus are similar to those in non-diabetic women if not corrected for maternal weight. However, when weight correction is made inhibin-A levels are significantly higher in diabetic women. The underlying explanation for this observation currently remains obscure and whether this elevation would exert any effects on screening performance in women with IDDM should inhibin-A be introduced as a prenatal marker for trisomy 21 has not assessed. It is suggested however that these observations may offer some new insights into the regulation of these proteins in normal and abnormal pregnancies.

Chapter Ten

Future Prospects

10.1 Review

Although the studies related in this thesis are all simple and observational in nature the data derived from them have considerably aided the understanding of inhibin biology in human pregnancy. In particular, it is now clear that the placenta is not the only significant source of inhibins in pregnancy but rather that the chorionic trophoblast is probably another source of some importance. Further, it would appear that these two sources are both differentially regulated and secrete different inhibins.

It has also been shown for the first time that the male term fetus produces inhibin-B, and it is possible that this inhibin has a role in the maturation of the male hypothalamo-pituitary-gonadal axis during *in utero* development. This is particularly exciting because in adults, recent studies have shown that inhibin-B promises to be a most sensitive marker of Sertoli cell function. The presence of adult levels of inhibin-B in the fetus therefore may offer an opportunity to explore more subtle effects of potential environmental testicular toxins than previously possible and to gain an improved understanding of normal and abnormal *in utero* testicular development.

The studies of inhibins in Down's syndrome have shown, for the first time, that maternal serum levels of inhibin-A are significantly elevated compared to normal and that this elevation is gestation dependent. Therefore, while it is likely that inhibin-A will become a valuable prenatal marker of Down's syndrome in the second trimester, adding approximately 10% to attainable detection rates, for a fixed false positive rate, it is most unlikely that it will be useful before 13 weeks of pregnancy. The precise gestation at which inhibin-A becomes a valuable marker has yet to be defined with precision, something that will probably only be described from prospective study of very large numbers of pregnancies.

Intriguingly, while inhibin-A levels in serum are elevated in Down's syndrome pregnancy it was shown that they are decreased in amniotic fluid. This data from this study suggest that for chorionic trophoblast, the abnormality in Down's syndrome may be expressed through the inhibin α-subunit. Studies are currently under way in the author's laboratory to explore this suggestion by quantifying inhibin subunit mRNAs in normal and Down's syndrome trophoblast, both placental and chorionic.

The detailed assessment of the different inhibin-A ELISA formats in the context of Down's syndrome screening was a useful exercise, affording reassurance about the newer formats. However, almost more importantly, the study also confirmed, albeit

indirectly, that maternal serum contains a large molecular weight forms of inhibin. The author is currently exploring this issue further (see below).

The small studies exploring inhibin-A levels in twin and diabetic pregnancies simply afforded data that will be important to screening laboratories should they wish to use inhibin-A as a prenatal marker in the future.

10.2 Future prospects

It remains unclear what inhibins are present in maternal serum throughout pregnancy and the author is currently exploring this in detail. It is hypothesised that with increasing gestation the placenta, and probably the chorion, changes the inhibins secreted reflecting changes in the relative amounts of mRNAs expressed. Further, the inhibin sizes secreted by trisomic pregnancies remain entirely unexplored. It remains possible that of the forms of inhibin-A detected by the ELISA used in these studies, only specific ones are secreted in abnormal amounts in Down's syndrome, forms that change in relative abundance across gestations. This hypothesis would explain why the relative levels of inhibin-A in Down's syndrome pregnancies change between the first and second trimester. If confirmed, then of course the implications for screening are significant, and would encourage the development of new antibodies against specific epitopes, allowing the detection of specific forms of inhibin-A.

It will be obvious that the studies described in this thesis are observational and "phenomenological" in nature. Within these pages, as is true elsewhere, very little consideration has been given to the possible roles for inhibins in pregnancy. As the understanding of inhibins in human pregnancy increases and the possible clinical applications become appreciated (Wallace and Healy 1996) it is clear that understanding function will become a priority. The development of transgenic animal strains, deficient in specific inhibins and activins will be a most important tool in this area. Indeed, such studies have already afforded important insights into roles of inhibins and activins in embryonic development. A transgenic mouse strain, with a mutated α-inhibin gene (Matzuk *et al* 1992) show normal embryogenesis, without any fetal abnormality, indicating that inhibin is not essential for developmental processes. (The mice do however, invariably develop malignant ovarian or adrenal tumours). Another transgenic strain (Vassalli *et al* 1994), mutant for the β_B-subunit (ie homozygotes have no active inhibin-B, activin-B and activin-AB) shows normal mesodermal development but late developmental abnormality (eyelid closure failure). Further, while the males exhibit normal fertility, the females do not labour

appropriately and do not have normal milk-ejection and their pups die. The delayed parturition is of extreme interest because activins have been implicated in human labour (as discussed in *Chapter One*) and given that preterm labour is the single most important cause of perinatal mortality (Creasey 1991) this area of perinatal research should now be a priority.

The development of other mutant strains will hopefully help to elucidate other important questions that remain, complementing human studies.

It is also clear that the functions of inhibins and activins differ fundamentally. While activins probably have multiple growth factor functions, inhibins appear to have traditional endocrine or paracrine roles in various tissues (Spencer *et al* 1992, Tuuri *et al* 1994) and it is likely that they have these functions in pregnancy. What these precise functions are however, remain obscure although it is likely that they may change with gestation. Studies exploring this area, addressing the regulation and effects of specific inhibins in pregnancy would now be most useful, dissecting out gestation effects.

Most recently, new inhibin subunits have been described and it remains to be reported whether they exist in humans and whether they form dimeric and biologically active proteins. Should this be confirmed then their roles, both as new activins and inhibins, in pregnancy will be intriguing. Likewise, the increasing understanding of inhibin binding proteins in pregnancy (Woodruff and Mather 1995, Woodruff *et al* 1997) may explain levels of regulation that are not apparent from simple circulating levels. Of course, the identification and cloning of the inhibin receptor is eagerly awaited and this event will likely afford opportunities not currently available.

In short, the increasing ability to detect specific inhibin and activin forms and to explore their biological functions in consort with their binding proteins promises exciting prospects for the application of inhibin to the management of human pregnancy.

Appendix One

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Appendix Two

Abbreviations

Abbrev.	Meaning	Abbrev.	Meaning
AF	Amniotic fuid	mRNA	Messenger ribonucleic acid
AFP	Alpha-fetoprotein	NIH	National Institute for Health
ANOVA	Analysis of variance	ns	Non-significant
CI	Confidence interval	PAPP-A	Pregnancy associated plasma protein
cDNA	complimentary de-oxyribonucleic acid	pNPP	p-nitrophenylphosphate
EDTA	Ethylenediamine tetra-acetic acid	PP14	Placental protein 14
EEC	Extra-embryonic coelom	RIA	Radioimmunoassay
EECF	Extra-embryonic coelomic fluid	RH	Recombinant human
ELISA	Enzme-linked immunosorbent assay	SAPU	Scottish antibody production unit
F β-hCG	Free beta-human chorionic gonadotrophin	SD	Standard deviation
FPR	False positive rate	SDS	Sodium dodecylusulphate
FSH	Follicle-stimulating hormone	SEM	Standard error of the mean
GnRH	Gonadotrophin releasing hormone	STD	Standard
hCG	human chorionic gonadotrophin	TGF-β	Transforming growth factor beta
IDDM	Insulin dependent diabetes mellitus		
IP	immunopurified		
IRMA	Immunoradiometric assay		
kDa	Kilo dalton		
lbs	pounds (weight)		
LH	Luteinising hormone		
MoM	Multiple of the median		
MS	Maternal serum		
NICHD	National Institute for Child Health and Human Development		

Appendix Three

Publications

(in chronological order)

Wallace EM, Harkness LM, Burns S, Liston WA.
Evaluation of maternal serum immunoreactive inhibin as a first trimester marker of Down's syndrome. *Clinical Endocrinology (Oxf)* 1994; **41**:483-486.

Wallace EM, Swanston IA, Groome NP.
Evaluation of maternal serum dimeric inhibin A as a first trimester marker of Down's syndrome. *Prenatal Diagnosis* 1995;**15**:359-362.

Wallace EM, Swanston IA, McNeilly AS, Ashby JP, Blundell G, Calder AA, Groome NP. Second trimester screening for Down's syndrome using maternal serum dimeric inhibin A. *Clinical Endocrinology (Oxf)* 1996;**44**:17-21.

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Second trimester dimeric inhibin-A in Down's Syndrome screening. *Prenatal Diagnosis* 1996;**16**:1101-1110

Riley SC, Wathen N, Chard T, Groome NP, Wallace EM.
Inhibins in extra-embryonic coelomic and amniotic fluids and maternal serum in early pregnancy. *Human Reproduction* 1996;**11**:2722-2776

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Inhibin-A in amniotic fluid in chromosomally normal and Down's syndrome pregnancies. *Journal of Endocrinology* 1997;**152**:109-112

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Noble PL, Wallace EM, Snijders RJM, Groome NP, Nikolaides KH. Maternal serum inhibin-A and free β-hCG concentrations in trisomy 21 pregnancies at 10 to 14 weeks of gestation. *British Journal of Obstetrics and Gynaecology* 1997;**104**:367-371

Clinical Endocrinology (1994) **41**, 483–486

Evaluation of maternal serum immunoreactive inhibin as a first trimester marker of Down's syndrome

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Summary

BACKGROUND AND OBJECTIVE Maternal serum immunoreactive inhibin has been shown to be significantly elevated in Down's affected pregnancies in the second trimester, suggesting that it may be useful in prenatal diagnosis. We have investigated whether it is similarly elevated in the first trimester.

DESIGN Stored maternal sera from women with Down's affected pregnancies and chromosomally normal control pregnancies were retrieved for analysis. These sera had been collected prospectively at either 11 or 12 weeks gestation as a routine antenatal booking procedure.

SUBJECTS From records, 11 women were identified as having had a Down's pregnancy. For each of these, 4 controls matched for gestation and duration-of-storage were also identified.

MEASUREMENTS Two different inhibin immunoassays were evaluated, one using an antibody raised against 31 kDa bovine inhibin and the other, a commercial two-site assay, using two antibodies directed against two distinct α-subunit epitopes.

RESULTS Neither assay detected a significant effect of gestation on serum inhibin levels. After combining the data from both gestations, no significant difference between the Down's samples and controls for either assay was detected. However, analysis of the data for each gestation separately revealed that one assay detected a significant difference in inhibin levels between Down's affected and unaffected pregnancies at 11 weeks gestation (mean $\pm$ SEM 3186 $\pm$ 195 *vs* 2020 $\pm$ 172 ng/l, $P < 0{\cdot}01$) but not at 12 weeks. The other, commercial, assay did not detect a significant difference at either gestation. In addition, there was poor association between the results of the two assays.

CONCLUSIONS These data suggest that immunoreactive inhibin, as detected by these assays, will not be useful as a late first trimester marker for Down's syndrome and also that these two assays detect different inhibin species in pregnancy serum.

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Prenatal screening for Down's syndrome has become an important and established part of modern antenatal care. At present, most screening programmes depend upon maternal age in combination with the measurement of human chorionic gonadotrophin (hCG) and α-fetoprotein (AFP), with or without unconjugated oestriol (uE_3), in maternal serum at 16 weeks gestation. Such an approach will detect approximately 65% of Down's affected pregnancies for an amniocentesis rate of 5% (RCOG, 1993). However, it has long been hoped that improvements in screening might increase this detection rate, while minimizing the amniocentesis rate, and also allow testing to be performed earlier in pregnancy. To this end, it was recently reported that inhibin, a heterodimeric glycoprotein produced by various tissues including the placenta, may be useful as a serum marker for Down's syndrome in the second trimester (van Lith *et al.*, 1992, Spencer *et al.*, 1993). In addition, a number of fetal and placental proteins have undergone initial evaluation of their usefulness as first, rather than second, trimester maternal serum markers for Down's syndrome (Macintosh & Chard, 1993 and references therein). We have therefore assessed inhibin as a potential maternal serum marker for Down's syndrome in the first trimester.

Subjects and methods

In Lothian, serum is routinely prospectively collected from all women at the first hospital antenatal visit and stored at $-20°C$. Eleven women (mean age $31{\cdot}6 \pm 5{\cdot}1$ years) were identified from records of known Down's affected pregnancies (detected by maternal serum AFP at 16 weeks and subsequent amniocentesis), allowing their stored serum to be retrieved. The serum from 5 of these women had been collected at 11 completed weeks of gestation and the other 6 at 12 completed weeks. These gestations were calculated from first trimester ultrasound scans, performed on the day of sampling. Similarly, for each Down's affected sample 4

control women, matched for gestation (ultrasound determined) and duration of storage, were identified (mean age 27·5 ± 4·9 years) and their samples retrieved.

Hormone assays

Inhibin was assayed using two different assays. First, a heterologous radioimmunoassay using an antibody (1989) raised against 31 kDa bovine inhibin and a tracer of iodinated 31 kDa bovine inhibin was performed (McLachlan *et al.*, 1987). Recombinant human inhibin A (rhINH-R-90/1) was used for standards with the results expressed as ng/l. The sensitivity of the assay was 780 ng/l and the coefficients of variation of intra-assay and interassay were 6·6 and 11·5% respectively. This assay is now distributed by the National Institute of Child Health and Human Development (NICHD).

Secondly, a commercial solid-phase two-site immunoenzymatic assay (Medgenex, High Wycombe, UK) was used to assay the same samples. The two antibodies employed in this assay were directed against distinct epitopes of the α-subunit of human inhibin. The standards were prepared from purified human follicular fluid. One unit was defined as the average serum concentration of 20 healthy males and the results were expressed as U/ml. The sensitivity of the assay was 0·1 U/ml and the coefficients of variation of intra-assay and interassay were 1·9 and 8·9% respectively.

Statistical analysis

Neither assay gave normally distributed results. To allow statistical comparisons to be performed, square root and logarithmic transformations were used for the results derived from the NICHD and Medgenex assays respectively. Analysis of variance (ANOVA) was then performed on the transformed, normally distributed data to assess the effects of gestation and Down's syndrome on the maternal serum inhibin levels. Where significance was detected, a post-hoc analysis was performed.

Results

NICHD assay

The mean (± SEM) maternal serum immunoreactive inhibin at 11 and 12 weeks gestation in the Down's syndrome samples were 3186 ± 195 and 2517 ± 441 ng/l and in the controls 2020 ± 172 and 2561 ± 198 ng/l respectively. The mean immunoreactive inhibin levels at the two gestations were not significantly different for either group. Figure 1 shows the data from both gestations combined, revealing no significant difference between Down's syndrome and normal pregnancies (2821 ± 267 and 2317 ± 138 ng/l respectively, NS). However, analysing each gestation separately, at 11 weeks the mean serum immunoreactive inhibin levels in Down's syndrome and control pregnancies were 3186 ± 195 and 2020 ± 172 ng/l respectively (Fig. 2). The mean inhibin level at 11 weeks gestation was significantly higher in the Down's affected pregnancies when compared to the controls ($P < 0·01$). No significant differences existed at 12 weeks (2517 ± 441 and 2561 ± 198 ng/l) (Fig. 2).

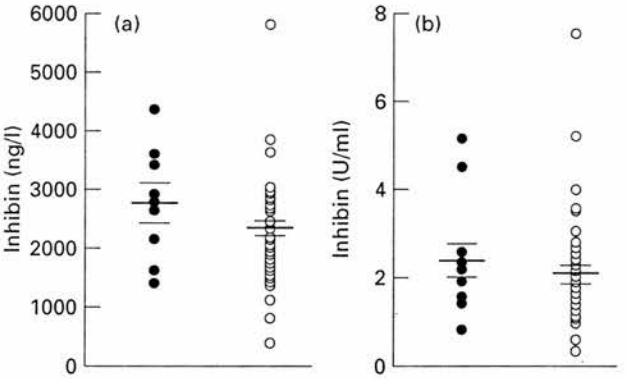

Fig. 1 Maternal serum immunoreactive inhibin levels in ●, Down's affected ($n = 11$) and ○, control ($n = 44$) pregnancies obtained using a, NICHD; b, Medgenex assays.

Medgenex assay

The mean (± SEM) maternal serum immunoreactive inhibin levels at 11 and 12 weeks gestation in the Down's syndrome samples were 2·46 ± 0·56 and 2·35 ± 0·62 U/ml and in the controls 1·84 ± 0·17 and 2·20 ± 0·33 U/ml respectively. The mean immunoreactive inhibin levels at the two gestations

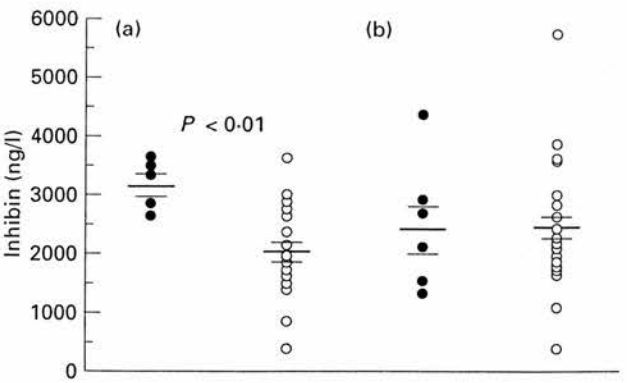

Fig. 2 Maternal serum immunoreactive inhibin levels (NICHD assay) in ●, Down's affected and ○, control pregnancies at a, 11 ($P < 0·01$ Down's *vs* controls) and b, 12 weeks gestation.

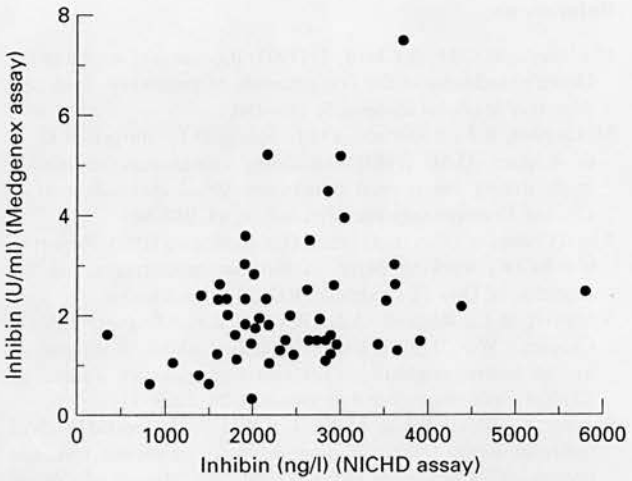

Fig. 3 Regression plot of maternal serum immunoreactive inhibin levels derived from Medgenex and NICHD assays. $r = 0{\cdot}28$; $P > 0{\cdot}5$.

were not significantly different for either group. Figure 1 shows the data from each gestation combined, revealing no significant difference between Down's syndrome and normal pregnancies ($2{\cdot}4 \pm 0{\cdot}4$ and $2{\cdot}04 \pm 0{\cdot}19$ U/ml respectively, NS). Analysis of each gestation separately also revealed no significant effect on inhibin levels exerted by Down's syndrome (data not shown).

Comparison between NICHD and Medgenex assays

There was poor correlation between the results derived from the two assays ($r = 0{\cdot}28$) (Fig. 3).

Discussion

Established screening programmes for Down's syndrome are currently timed for the second trimester of pregnancy (RCOG, 1993), utilizing maternal age in combination with the measurement of maternal serum hCG and AFP, with or without unconjugated oestriol (uE_3). More recently it was reported that inhibin may be a useful second trimester marker for Down's syndrome (van Lith *et al.*, 1992, Spencer *et al.*, 1993). Indeed, the data of Spencer *et al.* suggest that inhibin alone might offer a detection rate of 66% for a false positive rate of 5·3%. As reviewed by Macintosh and Chard (1993), a number of fetal and placental products have also been investigated as putative first trimester markers. We have therefore described the first data for inhibin as a marker for Down's syndrome in the first trimester, derived from two different assays for immunoreactive inhibin. These data show that, at 11 and 12 weeks gestation, no significant effect of gestation on maternal serum immunoreactive inhibin levels was demonstrated by either assay. Furthermore, when the data for these two gestations were combined, there was no significant difference between Down's affected and normal pregnancies in either assay. However, one of the assays detected a significant difference in maternal serum inhibin levels between Down's affected and normal pregnancies at 11 but not at 12 weeks gestation. In addition, our finding of a lack of correlation between the results of the two assays suggests that they are detecting different inhibin species in these pregnancy samples. Interestingly, we have not observed a similar disagreement between these two assays in samples from non-pregnant women (unpublished data) and so the current data may reflect changes in inhibin secretion specific to pregnancy. The NICHD assay detects free α-subunit and the related pro α-C fraction (Schneyer *et al.*, 1990) in addition to intact inhibin in serum. If in pregnancy the relative circulating concentrations of different inhibin species vary, compared to non-pregnancy, this might explain some of the differences. It was also reported recently that α_2-macroglobulin has been found to bind inhibin in peripheral plasma (Vaughan & Vale, 1993), altering immunoreactive detectable levels by masking different epitopes. Levels of α_2-macroglobulin rise by approximately 30% in pregnancy (Studd *et al.*, 1970) and it is conceivable that this could differentially interfere with antigen–antibody binding in one or other of these two assays.

Furthermore, while the isolated significant elevation in serum immunoreactive inhibin at 11 weeks, and not at 12 weeks, is surprising when the previous reports of screening in the second trimester are considered (van Lith *et al.*, 1992, Spencer *et al.*, 1993), these published second trimester results were obtained using the Medgenex assay. Our new finding of a lack of correlation between results derived from the two assays suggests that it is possible that the NICHD assay is detecting inhibin species, not detected by the Medgenex antibodies, that are elevated in Down's syndrome in the first trimester. Nonetheless, this isolated finding at 11 weeks gestation suggests that inhibin is unlikely to be a useful marker.

Importantly, we did not observe any storage related trends in our sample results derived from either assay (results not shown) suggesting that degradation changes were not responsible for artefactual differences. Furthermore, the samples were control-matched for storage.

In summary, this is the first report of first trimester maternal serum immunoreactive inhibin levels in Down's affected pregnancies using both these assays. Although a significant elevation in circulating maternal serum immuno-

reactive inhibin in Down's affected pregnancy at 11 weeks gestation compared to normal pregnancy was detected by a radioimmunoassay utilizing an antibody against 31 kDa bovine inhibin, this was not apparent at 12 weeks gestation. Also, using a different two-site assay (with both antibodies directed against α-subunit epitopes) no significant elevation was apparent. It also appears that these two assays do not detect identical inhibin species in pregnancy, as suggested by the poor correlation of results between the assays. We suggest that these data do not confirm inhibin, as detected by these two assays, as a useful marker for Down's syndrome in the first trimester. However, the results at 11 weeks gestation should encourage the assessment of the assays at other, earlier gestations. The remaining uncertainty about which inhibin proteins are being detected in pregnancy also merits the evaluation of other inhibin assays in the detection of Down's syndrome in the first trimester, in particular, those specific for dimeric inhibin or the β-subunit.

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EVALUATION OF MATERNAL SERUM DIMERIC INHIBIN A AS A FIRST-TRIMESTER MARKER OF DOWN'S SYNDROME

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SUMMARY

While second-trimester prenatal screening programmes for Down's syndrome have become established in prenatal care, it would be advantageous to be able to offer screening in earlier pregnancy. To this end, we have evaluated a new potential maternal serum marker, dimeric inhibin A, as a possible first-trimester marker. Dimeric inhibin A was measured in prospectively collected maternal serum from 23 cases of Down's syndrome and matched chromosomally normal controls, at 11–13 weeks' gestation. Levels of this protein were significantly elevated in the Down's pregnancies compared with the control pregnancies. The median multiple of the normal median (MOM) for the Down's samples was 2·46 (95 per cent confidence interval: 2·11–3·26, $P<0·0001$ vs. controls). These results suggest that dimeric inhibin A is a useful discriminator of Down's-affected pregnancies from normal pregnancies in the first trimester and that sensitive screening in combination with maternal age and other possible markers may be practicable in the first trimester.

KEY WORDS: Down's syndrome; inhibin; dimeric inhibin A; first trimester; maternal serum

INTRODUCTION

Down's syndrome (trisomy 21) is the single most common recognized cause of mental handicap. Not surprisingly therefore, prenatal screening for Down's syndrome has become an important and established part of modern prenatal care (Royal College of Obstetricians and Gynaecologists (RCOG), 1993). At present, screening programmes utilize maternal age in combination with the measurement of human chorionic gonadotrophin (hCG) and alpha-fetoprotein (AFP), with or without unconjugated oestriol (uE_3), in maternal serum at 15–20 weeks' gestation. Such an approach detects approximately 60–70 per cent of Down's-affected pregnancies with an amniocentesis rate, or approximate false-positive rate, of 5 per cent (RCOG, 1993). However, it would be desirable if sensitive and specific screening could be applied in the first trimester, affording earlier termination of pregnancy if indicated. To this end, a number of fetal and placental proteins have undergone initial evaluation of their usefulness as first-trimester maternal serum markers for Down's syndrome (Macintosh and Chard, 1993). Also, it has been recently reported that immunoreactive inhibin, a glycoprotein of placental origin in pregnancy (Abe *et al.*, 1990; Tovanabutra *et al.*, 1993), did not appear useful in first-trimester screening for Down's syndrome (van Lith *et al.*, 1994; Wallace *et al.*, 1994), although some potential value in the second trimester has been suggested (van Lith *et al.*, 1992; Spencer *et al.*, 1993; Cuckle *et al.*, 1994). We report here the successful application of a new sensitive assay specific for dimeric inhibin A in the detection of Down's syndrome in the first trimester of pregnancy.

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SUBJECTS AND METHODS

In Lothian, serum is routinely collected prospectively from all women at the first hospital prenatal visit and stored at −20°C. Twenty-three women (mean age 32·1 years, range 22–44 years) were identified from records of all known Down's-affected pregnancies, allowing their stored serum to be retrieved. The sera from eight of these women had been collected at 11 completed weeks of gestation, eight at 12 completed weeks, and seven at 13 completed weeks. These gestations had been calculated from ultrasound scans performed on the day of sampling. Similarly, for each Down's-affected sample four control women, matched for gestation (ultrasound-determined) and duration of storage, were identified and their samples retrieved (mean age 27·7 years, range 19–38 years). Three control samples, each one matched for three different 11-week Down's samples, were unable to be used due to insufficient sample volume, making 89 control samples available for assay.

The assay for dimeric inhibin A has been previously described (Groome and O'Brien, 1993) and validated for human serum (Groome *et al.*, 1994). Briefly, serum samples or standards are oxidized with 1 per cent (final w/v) hydrogen peroxide for 30 min prior to assay. Samples were diluted 1:4 prior to assay. The assay is an enzyme-linked two-site immunoassay using an immobilized anti-βA-inhibin subunit monoclonal antibody (E4) as a capture antibody. This is coupled covalently through Fc carbohydrate residues to treated hydrazide microplates (Avidplate-HZ). The Fab fraction of a mouse anti-α-inhibin subunit monoclonal antibody (R1), conjugated to alkaline phosphatase, is then used to bind to the α-subunit of the captured dimeric inhibin. A sensitive amplified enzyme assay then allows detection. Recombinant 32 kD human inhibin A (Genentech) was used for standards with the results expressed as pg/ml. The sensitivity of the assay was 8 pg/ml and the intra-plate and inter-plate coefficients of variation were 2·5 and 7·0 per cent respectively.

RESULTS

The mean (95 per cent confidence interval (CI)) maternal serum immunoreactive dimeric inhibin A at 11, 12, and 13 weeks' gestation in the control samples was 389·5 (341·3–437·7) pg/ml, 341·3 (279·9–402·7) pg/ml, and 263·6 (218·7–308·4) pg/ml, respectively. There was no significant difference between the levels at 11 and 12 weeks ($P=0·054$, Mann–Whitney U-test) but the mean level at 13 weeks was significantly lower than that at 11 weeks ($P=0·0004$, Mann–Whitney U-test), though not 12 weeks ($P=0·055$). The MOMs for both Down's and controls were therefore calculated from the controls for each gestation separately. Figure 1 shows the Down's cases from the three gestations against the 10th, 50th, and 90th centiles of the control MOMs. The median (95 per cent CI) MOM for the Down's samples was 2·46 (2·11–3·26) ($P<0.0001$ vs. controls, Mann–Whitney U-test). Table I displays the number of affected and unaffected pregnancies at different MOMs. In this series, for a given false-positive rate of 4 per cent (4/89), 65 per cent (15/23) of the Down's cases were detected. Maternal serum inhibin A was not associated with maternal age ($r=1·0$, $P=0·49$).

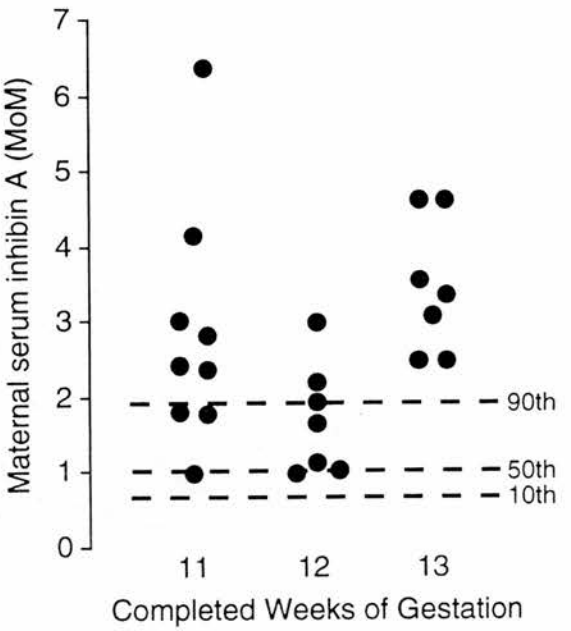

Fig. 1—The 10th, 50th, and 90th percentiles of maternal serum inhibin A MOMs of 89 chromosomally normal pregnancies with levels from 23 individual Down's-affected pregnancies

DISCUSSION

Inhibin is a heterodimeric glycoprotein, consisting of an α-subunit combined to one of two

Table I—Number (percentage) of affected and unaffected pregnancies with a given MOM above different arbitrary levels

MOM	No. (%) of affected pregnancies	No. (%) of unaffected pregnancies
0·5	23 (100)	86 (97)
1·0	22 (96)	42 (47)
1·5	19 (83)	18 (20)
2·0	15 (65)	6 (7)
2·5	10 (43)	1 (1)
3·0	9 (39)	1 (1)
3·5	5 (22)	0 (0)
4·0	4 (17)	0 (0)
4·5	3 (13)	0 (0)

β-subunits, β_A or β_B. Inhibin A is the dimer of the α-subunit and the β_A-subunit. In early pregnancy, inhibin is secreted from both the corpus luteum and the placenta, although the placenta becomes the major source after approximately 10–12 weeks' gestation (Abe *et al.*, 1990; Tovanabutra *et al.*, 1993), when peripheral plasma levels have reached a peak (Abe *et al.*, 1990).

The data presented suggest that maternal serum dimeric inhibin A may be a useful serum marker of Down's syndrome in the first trimester of pregnancy. For a given false-positive rate of 4 per cent in this series, 65 per cent of the Down's cases were detected with dimeric inhibin A. This compares very favourably with similar data obtained for free β-hCG in the first trimester (Spencer *et al.*, 1992; Aitken *et al.*, 1993). Figure 1 suggests that there may be a slight gestation effect, with better discrimination of Down's cases at 13 weeks than at 11 or 12 weeks. However, the number of cases reported is small and there is not a consistent trend across the three gestations. Further analysis of larger numbers will hopefully clarify this.

Maternal serum immunoreactive inhibin levels have previously been shown to be elevated in Down's-affected pregnancies in the second (van Lith *et al.*, 1992; Spencer *et al.*, 1993; Cuckle *et al.*, 1994) but not the first trimester (van Lith *et al.*, 1994; Wallace *et al.*, 1994). Importantly, the assays used in these studies utilize antibodies directed against epitopes only on the α-subunit of inhibin and so are unable to specifically detect intact dimeric inhibin. Indeed, it has been shown that such assays will detect inhibin α-subunit precursors in addition to free α-subunit and dimeric inhibin (Schneyer *et al.*, 1990). Recently, it was also reported that there was a poor correlation between immunoreactive inhibin levels measured in the same pregnancy samples by two different assays utilizing anti-α-subunit antibodies (Wallace *et al.*, 1994). This would suggest that these assays were detecting relatively different amounts of the various inhibin species circulating free in peripheral blood. This phenomenon might also explain some of the heterogeneity of results reported using one of these assays in the second trimester (Cuckle *et al.*, 1994). However, the novel assay reported here is specific for dimeric inhibin A and this is the first report of its application in pregnancy. As detailed above, recombinant human inhibin A is used for standards in the inhibin A assay and results are expressed as pg/ml. Of the other two inhibin assays previously reported, one (National Institute of Child Health and Human Development, U.S.A.) uses the same standard preparation while the other (Medgenix, Brussels) uses a partially purified form of inhibin derived from human follicular fluid ('human inhibin') expressed as U/ml (Wallace *et al.*, 1994). Considering the differences between the antibodies and standards used in the three inhibin assays, a direct comparison of inhibin levels across the assays is not appropriate. Indeed, the lack of correlation between the results from the two non-specific inhibin assays would confirm this. Thus, it would appear from the limited and indirect data available at present that it is likely that the peripheral serum levels of only certain inhibin species, such as inhibin A, will be different in chromosomally normal and abnormal pregnancies. An understanding of the biology of inhibin and the development of assays specific for different inhibin species is therefore fundamental to future progress in this area.

In summary, these promising preliminary inhibin A data have important implications for the future development of first-trimester Down's syndrome screening programmes. Certainly, larger prospective studies, allowing full evaluation of this novel marker, in combination with maternal age and established markers, are now merited.

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Second trimester screening for Down's syndrome using maternal serum dimeric inhibin A

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Summary

BACKGROUND AND OBJECTIVE Prenatal maternal serum screening for Down's syndrome has become an important and established part of modern antenatal care. Previously it has been reported that non-specific immunoreactive inhibin may be useful in this context. Using a novel assay we have evaluated dimeric inhibin A as a possible second trimester marker of Down's syndrome.

METHODS From 1992–1993 records, stored sera from women with Down's affected pregnancies and chromosomally normal control pregnancies were identified and retrieved for analysis. These sera had been prospectively collected at 15, 16 and 17 weeks gestation.

SUBJECTS Records revealed 21 women who had had a Down's syndrome pregnancy and who also had serum available for analysis. Sera from 150 chromosomally normal controls, matched for gestation and duration of storage, were also retrieved

MEASUREMENTS Dimeric inhibin A was measured using a recently developed two-site enzyme-linked immunoassay. This employs a capture anti inhibin β_A-subunit monoclonal antibody, covalently bound to a microtitre plate and a second anti inhibin α-subunit antibody conjugated to alkaline phosphatase, allowing detection.

RESULTS The mean (95% CI) maternal serum dimeric inhibin A in the samples from control pregnancies was 237 (201·5–273·4) ng/l, 266·9 (235·4–298·5) ng/l and 207·2 (178·5–235·9) ng/l at 15, 16 and 17 weeks gestation respectively. Expressing the results from the Down's samples as multiples of the normal median (MoM), the median (95% CI) MoM was 2·6 (2·25–3·57), significantly higher than the controls ($P < 0·0001$, Mann–Whitney *U*-test). In the sample set tested, for a given false positive rate of 5·3% inhibin A alone afforded a detection rate of 62%, detecting cases previously undetected by routine screening.

CONCLUSIONS Dimeric inhibin A appears to be a promising new marker for the prenatal detection of Down's syndrome. Further prospective evaluation and assessment with other established markers would now be merited.

Down's syndrome (trisomy 21) is the single most common recognized cause of mental handicap in the United Kingdom. Not surprisingly therefore, prenatal screening for this condition is now a common and integral part of modern obstetric practice. Such screening utilizes a risk calculation based upon maternal age in combination with the measurement of human chorionic gonadotrophin (hCG), or free β-hCG, and α-fetoprotein (AFP), with or without unconjugated oestriol (uE_3), in maternal serum at 15–21 weeks gestation. Amniocentesis targeted by such screening will detect 60–70% of Down's affected pregnancies with an overall amniocentesis rate, or approximate false positive rate, of 5% (RCOG, 1993). Thirty to 40% of affected cases therefore remain undetected by this approach and, because amniocentesis carries an excess risk of miscarriage of approximately 1% (Tabor *et al.*, 1986), a significant number of normal pregnancies are iatrogenically lost. A variety of other screening methods have therefore been reported (Benacerraf *et al.*, 1985, Cuckle *et al.*, 1990, Macintosh & Chard, 1993). Recently, Van Lith *et al.* (1992) reported that immunoreactive inhibin may be useful as an additional maternal serum marker for Down's syndrome in the second trimester although this was not confirmed by others (Spencer *et al.*, 1993, Cuckle *et al.*, 1994). The development of a new sensitive assay for intact dimeric inhibin A has enabled us to evaluate this specific inhibin species, rather than non-specific immunoreactive inhibin, as a potential marker for Down's syndrome in the second trimester of pregnancy.

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Subjects and methods

In south-east Scotland prenatal screening for Down's syndrome is offered to all women booking antenatally with a singleton pregnancy. This is performed at 16–21 weeks gestation, using maternal age in combination with maternal serum hCG and AFP levels. An aliquot of each serum sample collected for this programme is routinely stored at −20°C. Of the known Down's affected pregnancies during 1992–1993 there were 21 women from whom stored serum was available and these sera were retrieved. All these women had been at 15, 16 or 17 completed weeks of pregnancy at the time of sampling, as calculated from a first or early second trimester ultrasound scan. Fourteen of the 21 (67%) had been detected prenatally by the screening programme. One hundred and fifty control samples from women with a chromosomally normal pregnancy matched for gestation (within one week as determined by ultrasound) and duration of storage, were identified and retrieved. Forty-five of the control samples were at 15 completed weeks, 55 at 16 completed weeks and 50 at 17 completed weeks of pregnancy.

Dimeric inhibin A assay

The assay for dimeric inhibin A has been previously described (Groome & O'Brien, 1993) and validated for human serum (Groome *et al.*, 1994). Briefly, serum samples (diluted 1:4) or standards are oxidized with 10% (w/v) hydrogen peroxide for 30 minutes prior to assay. The assay is an enzyme-linked two-site immunoassay using an immobilized anti inhibin β_A-subunit monoclonal antibody (E4) as a capture antibody. This is coupled covalently through Fc carbohydrate residues to treated hydrazide microplates (Avidplate-HZ). The Fab fraction of a mouse anti inhibin α-subunit monoclonal antibody, conjugated to alkaline phosphatase, is then used to bind to the α-subunit of the captured dimeric inhibin. A sensitive amplified enzyme assay (Ampak, Dako UK) affords detection. Recombinant 32-kDa human inhibin A (Genentech) was used for standards with the results expressed as ng l. The sensitivity of the assay was 8 ng l and the intraplate and interplate coefficients of variation were 2·5 and 7·0% respectively.

Statistical analysis

Absolute inhibin values for the control samples at each separate gestation were compared using a non-parametric test, the Mann–Whitney *U*-test. Since a significant difference across gestation of pregnancy was detected in the controls, the Down's samples and controls were expressed as multiples of the normal median (MoM) to allow non-parametric comparison between the two groups. Each MoM, for both control and Down's samples, was calculated from the normal median of the respective gestation. The MoMs for all the Down's samples and controls were then pooled separately allowing comparison. Median MoMs for the Down's samples were expressed with 95% confidence intervals. The software package Statview was used throughout.

Table 1 Mean (95% CI) maternal serum dimeric inhibin A levels in 150 chromosomally normal singleton pregnancies at 15–17 weeks gestation.

Week of gestation	*n*	Inhibin (ng/l)
15	45	237·0 (201·5–273·4)
16	55	266·9 (235·4–298·5)
17	50	207·2 (178·5–235·9)

Results

The maternal serum levels of dimeric inhibin A in the chromosomally normal pregnancies are reported as means and 95% confidence intervals (CI) for each gestation

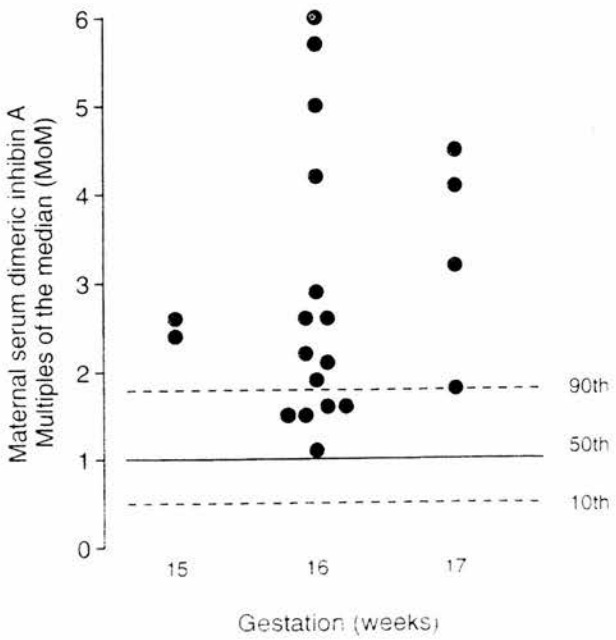

Fig. 1 The 10th, 50th and 90th percentiles of maternal serum inhibin A MoMs of 150 chromosomally normal pregnancies with levels from 21 individual Down's syndrome pregnancies.

Table 2 Number (percentage) of affected and unaffected pregnancies with a given MoM above different arbitrary levels

MoM	Number (%) of affected pregnancies	Number (%) of unaffected pregnancies
0·5	21 (100)	135 (90)
1·0	21 (100)	76 (51)
1·5	21 (100)	28 (19)
2·0	14 (67)	11 (7)
2·5	11 (52)	4 (3)
3·0	7 (33)	1 (1)
3·5	6 (29)	0 (0)
4·0	6 (29)	0 (0)
4·5	4 (19)	0 (0)

separately (Table 1). The level at 17 weeks gestation was significantly lower than that at 16 weeks ($P < 0{\cdot}02$, Mann–Whitney U-test). There were no other significant differences.

In all the Down's syndrome samples the dimeric inhibin levels were above the normal median (Fig. 1). The median (95% CI) MoM was 2·6 (2·25–3·57) which was significantly higher than the controls ($P < 0{\cdot}0001$, Mann–Whitney U-test). The inhibin data fitted a log Gaussian frequency distribution with standard deviation of $\log_{10}$ inhibin of 0·27 in the controls and 0·20 in the Down's syndrome cases. The mean $\log_{10}$ inhibin A in the Down's syndrome cases was 0·41. For a given false positive rate (FPR) of 5·3% (8/150), 62% (13/21) of the Down's syndrome samples would be detected at a cut-off MoM of 2·2. Table 2 shows the detection rates and corresponding FPRs at different arbitrary MoMs.

Of the seven Down's samples that had been undetected by the routine screening programme two had dimeric inhibin A levels above 2·2 MoM while three Down's samples with levels below this arbitrary detection limit had been detected by screening (Table 3).

Table 3 Maternal serum dimeric inhibin A levels (MoM) in 21 Down's affected pregnancies compared with the result from routine screening

Dimeric inhibin A level (MoM)	Detected/undetected by standard screening
6·0	undetected
5·7	detected
5·0	detected
4·5	detected
4·2	detected
4·1	detected
3·2	detected
2·9	undetected
2·6	detected
2·6	detected
2·6	detected
2·4	detected
2·2	detected
2·1	detected
1·9	undetected
1·8	undetected
1·6	undetected
1·6	detected
1·5	undetected
1·5	detected
1·1	undetected

Discussion

Inhibin is a heterodimeric glycoprotein, consisting of an α-subunit combined to one of two β-subunits, β_A or β_B. Inhibin A is the dimer of the α and β_A subunits. The placenta is the major source of inhibin during pregnancy (Abe *et al.* 1990, Tovanabutra *et al.*, 1993). Maternal serum inhibin levels have previously been shown to be elevated in Down's affected pregnancies in the second trimester (Van Lith *et al.*, 1992; Spencer *et al.*, 1993; Cuckle *et al.*, 1994). However, the absolute inhibin levels in the controls and the degree of elevation in the Down's cases reported in these three studies varied so considerably that Cuckle *et al.* (1994) suggested that immunoreactive inhibin, as detected, was unlikely to prove a valuable marker of Down's syndrome. All these previous reports relate to a commercial assay that utilizes two distinct antibodies, both directed against the α-subunit of inhibin (Medgenix, UK).

There are a number of possible inhibin species circulating in peripheral blood including the two 31-kDa dimeric forms (inhibin A and inhibin B), larger dimeric forms (58–73kDa), large free precursor α-subunits and the free processed α-subunit (Robertson *et al.*, 1995). By the nature of the antibodies employed, the Medgenix assay is unable to specifically detect intact dimeric inhibin A but probably also detects free inhibin α-subunits and the related pro α-C and pro α-C–pro α-N fractions (Schneyer *et al.*, 1990). It is possible that this non-specificity may account for the very heterogeneity of the results that has caused some concern (Cuckle *et al.*, 1994). Further, both Spencer *et al.* (1993) and Cuckle *et al.* (1994) noted that the distribution of levels of inhibin detected by the Medgenix assay in pregnancy was wide. This would also argue against inhibin being likely to be a valued marker of Down's syndrome. Recently it was reported that two immunoassays, both utilizing anti inhibin α-subunit antibodies, appeared to recognize different inhibin species in the same pregnancy samples, possibly due to

differing antibody affinities for different species (Wallace *et al.*, 1994). Further, serum factors that are known to interfere with immunoreactive inhibin detection (Vaughan & Vale, 1993) increase in pregnancy (Studd *et al.*, 1970). It is clear therefore that the usefulness of inhibin in prenatal screening for Down's syndrome may crucially depend on which inhibin species are being detected and thus on the antibodies utilized in the assay(s) applied. Indeed, while non-specific immunoreactive inhibin has not been shown to be a good marker for Down's syndrome in the first trimester (Van Lith *et al.*, 1994; Wallace *et al.*, 1994), dimeric inhibin A has been shown to be significantly elevated in Down's syndrome pregnancies at this gestation (Wallace *et al.*, 1995). In this regard it is important that the data reported here relate specifically to intact dimeric inhibin A species. The assay used displays no cross reactivity with inhibin B, or free subunits or their free precursors (N. P. Groome, unpublished data).

Despite the wide distribution of inhibin noted previously, however, using the Medgenix inhibin assay Van Lith *et al.* (1992) reported a preliminary detection rate of 40% with a false positive rate of 5% and a median MoM (95%CI) of 1·3 (0·9–1·9) for inhibin alone across a gestational range of 14–18 weeks. Our corresponding detection rate of 62% for 5·3% and median MoM (95% CI) of 2·6 (2·25–3·57) for intact dimeric inhibin A at 15–17 weeks gestation would appear to compare very favourably with non-specific immunoreactive inhibin. Also, in the series reported here the standard deviations of $\log_{10}$ inhibin in both the controls and the Down's cases show that inhibin A levels are less widely distributed than those for non-specific immunoreactive inhibin previously reported. This phenomenon could explain the improved detection performance of intact dimeric inhibin A relative to immunoreactive inhibin. It is also possible that only some of the circulating inhibin species are discriminatory with regard to Down's syndrome. If the less specific assays were detecting some of the non-discriminatory inhibin species, undetected by the dimeric assay, then poorer detection performance would be observed. However, until the processed free α-subunit, both dimers and circulating precursors can all be detected specifically and separately this remains speculation.

Finally, the finding that dimeric inhibin A detected Down's affected samples that had previously screened negative with traditional markers suggests that this inhibin species may have a useful role as an adjuvant marker in the second trimester, increasing detection rates. Further assessment of inhibin A in combination with other established markers will clarify this but it is clear that these novel data may have important implications for future screening programmes. We therefore propose that further prospective assessment of this new marker in the second trimester is now required.

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DIMERIC INHIBIN A AS A MARKER FOR DOWN'S SYNDROME IN EARLY PREGNANCY

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Abstract *Background.* In screening for Down's syndrome in the second trimester of pregnancy, the concentrations of alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and intact human chorionic gonadotropin in maternal serum are widely used markers. We investigated a new marker, dimeric inhibin A, and compared its predictive value with that of the established markers.

Methods. Serum samples were obtained at 7 to 18 weeks of gestation from 58 women whose fetuses were known to be affected by Down's syndrome, 32 whose fetuses were affected by trisomy 18, and 438 whose fetuses were normal, and the samples were analyzed for each marker. Individual serum concentrations of each marker were converted to multiples of the median value at the appropriate length of gestation in the women with normal pregnancies, and rates of detection of Down's syndrome by screening for inhibin A in various combinations with the other markers were estimated by multivariate analysis.

Results. In the women with fetuses affected by Down's syndrome, the serum inhibin A concentrations were 2.06 times the median value in the women with normal pregnancies ($P<0.001$). This compared with 2.00 times the median for the β subunit of human chorionic gonadotropin, 1.82 times the median for intact human chorionic gonadotropin, and 0.72 times the median for alpha-fetoprotein. The serum concentrations of inhibin A in the women with fetuses affected by Down's syndrome did not appear to be significantly elevated above normal until the end of the first trimester and were not significantly different from normal in the women with fetuses affected by trisomy 18 ($P=0.17$). The rate of detection of Down's syndrome was 53 percent and the false positive rate was 5 percent when alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age were used together as predictors. The detection rate increased to 75 percent when inhibin A was added ($P=0.002$).

Conclusions. In the second trimester of pregnancy, measuring inhibin A in maternal serum, in combination with measurements of alpha-fetoprotein and the β subunit of human chorionic gonadotropin, significantly improved the rate of detection of Down's syndrome. (N Engl J Med 1996;334:1231-6.)

THE sensitivity and specificity of screening for Down's syndrome have improved in recent years with the identification of new biochemical markers of this disorder. Since the observation that serum levels of alpha-fetoprotein were reduced in women with fetuses affected by chromosomal abnormalities,[1] numerous other fetoplacental markers in maternal serum have been found to have altered levels in pregnancies with fetuses affected by aneuploidy. The most useful markers for screening in the second trimester are intact human chorionic gonadotropin,[2] the β subunit of human chorionic gonadotropin,[3,4] unconjugated estriol,[5,6] and alpha-fetoprotein.[7,8] The serum levels of all these markers overlap in affected and unaffected populations, however, and so the odds that a particular value is associated with an affected pregnancy are used to modify the a priori risk specific to maternal age.[9] The most effective approach to screening is to use combinations of markers (taking into account correlations between markers), and most protocols use alpha-fetoprotein and either intact human chorionic gonadotropin or its β subunit, with[10] or without[11,12] unconjugated estriol. In clinical practice, approximately two thirds of pregnancies in which the fetuses are affected by Down's syndrome can be detected in the 3 to 5 percent of the screened population with the highest risk.[13-17] Fetuses affected by trisomy 18, which is associated with very low levels of intact human chorionic gonadotropin and its β subunit, may also be identified with the use of a separate protocol.[18,19]

Efforts to improve biochemical screening further have centered on the investigation of screening in the first trimester and on the search for better markers. The performance of certain markers varies between the first and the second trimesters. For example, intact human chorionic gonadotropin, a widely used marker in the second trimester, is a poor marker in the first,[20,21] whereas the β subunit of human chorionic gonadotropin is a useful marker during both stages of pregnancy.[21,22] Recent investigations of inhibin, a dimeric glycoprotein of placental origin composed of one α and one βA or βB subunit, found elevated serum levels in women in the second trimester of pregnancy whose fetuses were affected by Down's syndrome[23-25] but no differences between women with such pregnancies and those with unaffected pregnancies during the first trimester.[26,27] These studies used an enzyme immunoassay with antibodies directed against epitopes on the inhibin α subunit. Further studies using a new assay specific for dimeric inhibin A (α-βA) have suggested a possible role for dimeric inhibin A as a screening marker, since elevated serum levels have been found in women with fetuses affected by Down's syndrome in both the first and the second trimesters.[28,29]

In this study, we extended the investigation of inhibin A to a larger group of women with fetuses affected by Down's syndrome or trisomy 18 and compared its

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performance in screening with that of the established markers alpha-fetoprotein, intact human chorionic gonadotropin, and the β subunit of human chorionic gonadotropin.

METHODS

Maternal Serum Samples

All aliquots of maternal serum used in this study came from samples collected between 1987 and 1994 by the prenatal screening service in western Scotland. To avert repeated freeze-and-thaw cycles, which might affect marker levels in future studies, frozen serum samples from women whose fetuses were confirmed as being chromosomally abnormal were routinely retrieved from storage, thawed, divided into aliquots, and returned to a freezer bank for long-term storage pending further analyses. A total of 528 samples were analyzed, including 58 samples from women whose fetuses had Down's syndrome, 32 samples from women whose fetuses had trisomy 18, and 438 control samples from women with unaffected pregnancies at 7 to 18 weeks' gestation. For each trisomic sample, three to six control samples were selected and matched for the same completed week of gestation and length of time in frozen storage (within six months). Additional controls, selected on the basis of length of gestation, were added to supplement the small number of matched serum samples collected earlier in gestation. Fourteen of the Down's syndrome samples, which were collected at 7 to 14 weeks' gestation, were part of a series previously investigated for alpha-fetoprotein, unconjugated estriol, the β subunit of human chorionic gonadotropin, and pregnancy-associated plasma protein A.[21,30] The remaining 44 Down's syndrome samples were collected between 15 and 18 weeks' gestation; 35 of these were part of a previous series of 37 reported in a prospective study.[16] Four of the trisomy-18 samples collected at 8 to 12 weeks' gestation were also included in previous studies.[21,30] The remaining 28 trisomy-18 samples were collected at 15 to 18 weeks' gestation. The length of gestation was estimated by calculating the number of completed weeks from the date of the last menstrual period or by ultrasound scanning. If two different estimates were obtained for the same pregnancy, reliable information on the menstrual cycle was used as the primary determinant, unless the ultrasound estimate was one or more completed weeks greater than or two or more completed weeks less than the length of gestation as determined from the last menstrual period, in which case the ultrasound estimate was used.[16]

Marker Analyses

Serum dimeric inhibin A was measured with a two-site enzyme-linked immunosorbent assay (ELISA) that uses an immobilized monoclonal antibody (E4) against the βA subunit of inhibin as the capture antibody. This was coupled covalently through Fc carbohydrate residues to treated hydrazide microplates (Avidplate-HZ, UniSyn Technologies, Tustin, Calif.).[31,32] The Fab fraction of a mouse monoclonal antibody (R1) against the α subunit of inhibin, conjugated to alkaline phosphatase, was then used to bind the α subunit of the captured dimeric inhibin. A sensitive amplified-enzyme assay (Ampak, Dako, High Wycombe, United Kingdom) allowed detection of the bound second antibody. Recombinant 32-kd human inhibin A (Genentech, South San Francisco) was used for standards, with results expressed in picograms per milliliter. The intraplate and interassay coefficients of variation were 2.5 percent and 7.0 percent, respectively, and assay sensitivity was 8 pg per milliliter.

The β subunit of human chorionic gonadotropin was measured by ELISA as previously described.[33] Intact human chorionic gonadotropin was measured with the use of a commercial immunoradiometric assay (MAIA Clone, Serono, Biodata, Rome, Italy), after a 1:500 dilution of samples.[16] Alpha-fetoprotein was measured with the use of a sensitive immunoradiometric assay developed in our institution.[21]

Aliquots of serum samples from all the selected women were obtained from frozen storage in Glasgow, coded, randomized, and shipped in dry ice to the Centre for Reproductive Biology in Edinburgh to be assayed for inhibin A. All other analyses of markers were performed in Glasgow. Alpha-fetoprotein and intact human chorionic gonadotropin were measured prospectively as part of the routine screening program in the second-trimester samples[16] and retrospectively in the first-trimester samples.[21] The β subunit of human chorionic gonadotropin was measured in all samples retrospectively. All data were collated in Glasgow, and sample decoding and data analysis were performed only after all assays had been completed.

Table 1. Serum Concentrations of Inhibin A, Intact Human Chorionic Gonadotropin, the β Subunit of Human Chorionic Gonadotropin, and Alpha-Fetoprotein in Control Women and Women with Pregnancies Affected by Down's Syndrome at Various Lengths of Gestation.*

Marker	Gestational Range (weeks)	Controls†	Down's Syndrome	P Value‡
		multiples of the median (no. of women)		
Inhibin A	7–11	1.00 (148)	0.98 (8)	0.68
	13–14	1.00 (58)	2.60 (6)	0.002
	15–18	1.00 (202)	2.24 (44)	<0.001
Combined	7–18	1.00 (438)	2.06 (58)	<0.001
Intact human chorionic gonadotropin	7–11	1.08 (148)	0.95 (8)	0.62
	13–14	0.93 (58)	1.19 (6)	0.09
	15–18	1.03 (202)	2.04 (44)	<0.001
Combined	7–18	1.03 (438)	1.82 (58)	<0.001
β Subunit of human chorionic gonadotropin	7–11	1.00 (140)	1.79 (8)	0.02
	13–14	0.94 (58)	2.15 (6)	0.002
	15–18	1.03 (112)	2.05 (44)	<0.001
Combined	7–18	1.00 (340)	2.00 (58)	<0.001
Alpha-fetoprotein	7–11	0.99 (148)	0.70 (8)	0.10
	13–14	0.96 (58)	0.59 (6)	0.02
	15–18	0.95 (202)	0.74 (44)	<0.001
Combined	7–18	0.95 (438)	0.72 (58)	<0.001

*Values are expressed in multiples of the median among the women with normal pregnancies.

†Combined numbers include 30 controls studied at 12 weeks' gestation. The controls studied for the β subunit of human chorionic gonadotropin were a subgroup of the 438 controls.

‡P values were determined by the Mann–Whitney test.

Statistical Analysis

For inhibin A, the median level at each completed week of gestation was calculated from the results of the analyses in the control women's serum samples and used to convert individual marker values to multiples of the normal gestational median. Multiples of the median for alpha-fetoprotein, intact human chorionic gonadotropin, and the β subunit of human chorionic gonadotropin were calculated with the use of medians previously determined in a larger series of women with normal pregnancies from which the 438 controls were selected (Table 1). Goodness of fit to log gaussian distributions for the marker values in the women with normal fetuses and in those with chromosomally abnormal fetuses was assessed by probability plot and the Kolmogorov–Smirnov test. The measures of the distribution (means and standard deviations) for each marker in the control women and in women whose fetuses were affected by Down's syndrome were calculated by taking the $\log_{10}$ of the median as the mean and the difference between the 10th and 90th percentiles in logs divided by 2.56 as the standard deviation.[7] Correlations between pairs of markers were estimated with the use of log-transformed values.

Detection rates for Down's syndrome and corresponding false positive rates were calculated with gaussian models of the distribution of likelihood ratios and the age distribution of pregnancies in western Scotland. First, the proportion of fetuses affected by Down's syndrome that would be expected at each individual maternal age was calculated from the age-related risk[8] and the proportion of pregnancies at each age in the population. Next, likelihood ratios were calculated for each marker and combination of markers for all samples from women with fetuses affected by Down's syndrome and from the control women with the use of the variables derived in this study.[9] A specific risk threshold was then selected to define a high-risk group, and cutoff likelihood ratios were calculated from the age-specific risks. The distribution of likelihood ratios for the samples from women whose fetuses had Down's syndrome was then used to calculate the predicted detection rate at each maternal age. This detection rate was

multiplied by the proportion of fetuses affected by Down's syndrome expected for that age, and the overall detection rate was obtained by summation. Similarly, the corresponding false positive rates at each threshold risk were obtained with the use of the distribution of likelihood ratios for the control samples and the proportion of pregnancies at each individual maternal age in the population of pregnant women in western Scotland.

RESULTS

Serum concentrations of inhibin A in women with normal pregnancies rise to a median of about 550 pg per milliliter at 8 to 9 weeks' gestation, followed by a decline that levels out at about 180 pg per milliliter at 15 weeks' gestation. The serum levels of inhibin A were elevated in the women whose fetuses were affected by Down's syndrome, with an overall median value 2.06 times the median among the women with normal pregnancies (by the Mann–Whitney test, $P<0.001$). The distribution of results according to length of gestation (Fig. 1) shows a rising trend, from a median value 0.98 times the median among the women with normal pregnancies at 7 to 11 weeks' gestation ($P=0.68$) to 2.24 times the median among the women with normal pregnancies at 15 to 18 weeks' gestation ($P<0.001$) (Table 1). This trend appears similar to that for intact human chorionic gonadotropin but contrasts with that for the β subunit of human chorionic gonadotropin, which is associated with elevated levels in women with fetuses affected by Down's syndrome both early and later in gestation (Table 1). Alpha-fetoprotein levels are reduced in the first and second trimesters in women with fetuses affected by Down's syndrome.

The means and standard deviations of the log gaussian distributions for inhibin A, intact human chorionic gonadotropin, the β subunit of human chorionic gonadotropin, and alpha-fetoprotein are summarized in Table 2. The goodness of fit for inhibin A in women with fetuses affected by Down's syndrome and in control women at 15 to 18 weeks of gestation was examined with the use of probability plots. Neither log-transformed nor untransformed values gave a perfectly straight line, but the fit to the log gaussian distributions was better, as assessed by the Kolmogorov–Smirnov test ($P=0.51$ for Down's syndrome; $P=0.06$ for normal pregnancies), and subsequent statistical analyses were based on the assumption of log normality. Previous studies of alpha-fetoprotein, intact human chorionic gonadotropin, and the β subunit of human chorionic gonadotropin have shown that these markers fit log gaussian distributions.[8,11,12]

Correlation coefficients for inhibin A with the β subunit of chorionic gonadotropin, intact human chorionic gonadotropin, alpha-fetoprotein, and maternal age both in women with normal pregnancies and in those with fetuses affected by Down's syndrome are summarized in Table 3. The strongest correlation was with intact human chorionic gonadotropin; there was no significant correlation between inhibin A and the β subunit of human chorionic gonadotropin in the second trimester in either the control women ($P=0.11$) or those with fetuses affected by Down's syndrome

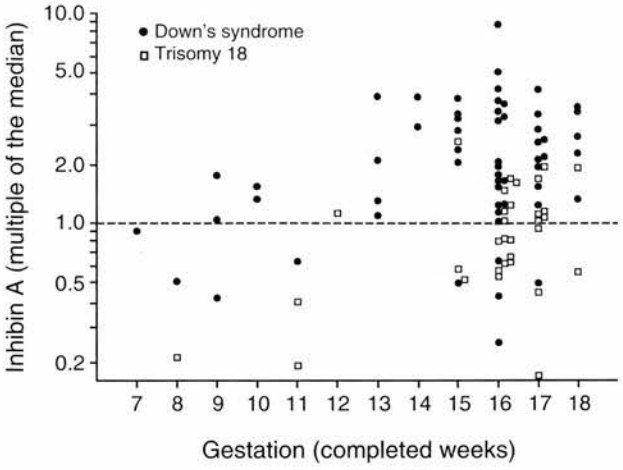

Figure 1. Serum Concentrations of Inhibin A, Expressed in Multiples of the Median Value in Women with Normal Pregnancies, in 58 Pregnant Women with Fetuses Affected by Down's Syndrome and 32 with Fetuses Affected by Trisomy 18 at 7 to 18 Weeks' Gestation.

($P=0.13$). Intact human chorionic gonadotropin and the β subunit of human chorionic gonadotropin were strongly correlated.

Using the statistical characteristics of the distributions of each marker in the control women and in those whose fetuses were affected by Down's syndrome at 15 to 18 weeks' gestation, we calculated detection rates at a fixed 5 percent false positive rate for each marker in combination with maternal age, and for various groups of markers in combination with maternal age, in a typical screened population (Table 4). We obtained the highest predicted detection rate (75 percent at a 5 percent false positive rate) by adding inhibin A to the combination of alpha-fetoprotein and the β subunit of human chorionic gonadotropin. This protocol resulted in a 22 percent increase in the detection of Down's syndrome when tested against both the population model and the actual study cases (two-tailed $P=0.002$ by McNemar's test).

The serum concentrations of inhibin A in women whose fetuses were affected by trisomy 18 were slightly reduced at 0.84 times the median in the women with normal pregnancies ($P=0.17$). There were too few

Table 2. Measures of the Log Gaussian Distributions of Each Serum Marker, Expressed in Multiples of the Median Values in Samples from Control Women and Women with Pregnancies Affected by Down's Syndrome at 15 to 18 Weeks' Gestation.

MARKER	CONTROLS		DOWN'S SYNDROME	
	MEAN	SD	MEAN	SD
Inhibin A	0.0000	0.2967	0.3502	0.3521
Intact human chorionic gonadotropin	0.0128	0.2196	0.3086	0.2064
β Subunit of human chorionic gonadotropin	0.0128	0.2609	0.3188	0.3061
Alpha-fetoprotein	−0.0223	0.1609	−0.1337	0.1805

measurements at earlier gestational ages to permit examination of the values in relation to the length of gestation (Fig. 1), but for this chromosomal trisomy, inhibin A was clearly different from the other markers. The median values of intact human chorionic gonadotropin and its β subunit were 0.30 and 0.14 times the medians in the women with normal pregnancies, respectively ($P<0.001$), and for alpha-fetoprotein the median value was 0.53 times the median in the women with normal pregnancies ($P<0.001$).

Table 4. Detection Rates and 95 Percent Confidence Intervals (CI) for Down's Syndrome at a Constant 5 Percent False Positive Rate for Various Combinations of Serum Markers and Maternal Age.

Variables	Detection Rate (%)	95% CI
Alpha-fetoprotein and age	33	19–48
Intact human chorionic gonadotropin and age	41	26–57
β Subunit of human chorionic gonadotropin and age	47	32–63
Inhibin A and age	48	32–63
Alpha-fetoprotein, intact human chorionic gonadotropin, and age	54	38–69
Alpha-fetoprotein, β subunit of human chorionic gonadotropin, and age	53	37–68
Alpha-fetoprotein, inhibin A, and age	57	41–72
Intact human chorionic gonadotropin, inhibin A, and age	57	41–72
β Subunit of human chorionic gonadotropin, inhibin A, and age	68	52–81
Intact human chorionic gonadotropin, β subunit of human chorionic gonadotropin, and age	40	25–56
Alpha-fetoprotein, intact human chorionic gonadotropin, inhibin A, and age	72	57–84
Alpha-fetoprotein, β subunit of human chorionic gonadotropin, inhibin A, and age	75	60–87
Alpha-fetoprotein, intact human chorionic gonadotropin, β subunit of human chorionic gonadotropin, and age	52	36–67

Discussion

Inhibin is a heterodimeric glycoprotein with a molecular weight of 32,000, composed of one α subunit and one of two related β subunits (βA or βB). Inhibin A (α-βA) and inhibin B (α-βB) are synthesized by the gonads and regulate the secretion by the pituitary of follicle-stimulating hormone.[34] In addition to these mature dimers, forms of inhibin with greater molecular weights, representing partially processed or unprocessed dimers and processed or unprocessed free α subunits, circulate in the peripheral blood.[35] In pregnancy, the main source of inhibin secretion switches from the corpus luteum to the placenta,[36] and the level of immunoreactive and bioactive inhibin is significantly higher than that in nonpregnant women.[37,38]

Until recently, studies of immunoreactive inhibin in pregnancy have depended on assays using antibodies directed against epitopes only on the α subunit.[37,39] Such assays detect the mature dimers and all unprocessed or partially processed molecules containing the α subunit.[39] A commercially available assay (Medgenix, Brussels, Belgium) with this type of inhibin specificity has been used in three separate studies to measure inhibin levels in maternal serum from second-trimester pregnancies affected by Down's syndrome.[23-25] Inhibin values that were 1.9,[23] 3.6,[24] and 1.3[25] times the median values in the women with normal pregnancies were reported.

Inhibin levels in women with first-trimester pregnancies affected by Down's syndrome have also been investigated in two studies with the commercial assay. No differences were found between women with fetuses affected by trisomy 21 and control women.[26,27] In our study with a new assay specific for dimeric inhibin A,[31,32] the increase in the concentration of inhibin A associated with affected pregnancies did not occur until the end of the first trimester. Recently, however, significant increases in inhibin A as early as 11 weeks' gestation have been reported,[28] although there was some evidence of an effect of the length of gestation; there was a better rate of detection of Down's syndrome at 13 weeks than at 11 or 12 weeks. It is clear that larger numbers of samples are required to determine more accurately the levels of inhibin A in women with fetuses affected by Down's syndrome before 15 weeks' gestation. The greater increase in the serum concentration of inhibin A from women with fetuses affected by Down's syndrome later in gestation is similar to the trend noted previously for other placental markers, including intact human chorionic gonadotropin,[21] pregnancy-specific β_1 glycoprotein,[40,41] and pregnancy-associated plasma protein A.[30] The exception to this rule appears to be the β subunit of human chorionic gonadotropin, which

Table 3. Coefficients of Correlation (r) between Serum Inhibin A and Other Variables in Control Women and Women with Fetuses Affected by Down's Syndrome at 15 to 18 Weeks' Gestation.*

Variables	Controls		Down's Syndrome	
	r	P value	r	P value
Inhibin A and intact human chorionic gonadotropin	0.27	<0.001	0.39	0.008
Inhibin A and β subunit of human chorionic gonadotropin	0.15	0.11	0.23	0.13
Inhibin A and alpha-fetoprotein	0.24	0.001	0.24	0.11
Inhibin A and maternal age	−0.03	0.69	0.25	0.10
Intact human chorionic gonadotropin and β subunit of human chorionic gonadotropin	0.87	<0.001	0.76	<0.001
Intact human chorionic gonadotropin and alpha-fetoprotein	0.23	0.001	0.25	0.11
β Subunit of human chorionic gonadotropin and alpha-fetoprotein	0.15	0.12	0.20	0.19

*Correlation coefficients were calculated with the log multiple of the median for each variable.

is elevated in women with fetuses affected by Down's syndrome in both the first and the second trimesters,[21,42] although it is not known whether the increases are of the same magnitude throughout gestation.

Two studies of the same series of 19 women with second-trimester pregnancies affected by Down's syndrome[25,29] provide a comparison of the performance of the commercial (Medgenix) assay with that of the specific assay for dimeric inhibin A used in the present investigation. Overall median values 1.3 times the median value for immunoreactive inhibin[25] and 1.6 times the median value for dimeric inhibin A in the women with normal pregnancies[29] were obtained, suggesting that the latter is more specific for Down's syndrome. One other study using an inhibin assay with the same antibody specificity as our own reported a median value 1.88 times the control median value in 20 women with second-trimester pregnancies affected by Down's syndrome and a strong correlation between inhibin and intact human chorionic gonadotropin.[43]

In this study, we found a statistically significant 22 percent increase in the rate of detection of Down's syndrome when inhibin A was added to the established screening protocol including alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age. This compares with detection rates estimated at 54 percent for the combination of alpha-fetoprotein, intact human chorionic gonadotropin, and maternal age and 53 percent for the combination of alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age. These results are consistent with those of previous retrospective studies with respect to alpha-fetoprotein, intact human chorionic gonadotropin, and maternal age[10-12] but lower than those reported in some studies with respect to alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age.[3,12] The confidence limits of our estimates are wide, however, because of the small size of the sample.

The inclusion of inhibin A in multimarker screening protocols did not, however, contribute to the detection of trisomy 18. In contrast to the elevated levels found in women with second-trimester pregnancies affected by Down's syndrome, levels of inhibin A associated with trisomy 18 were not significantly different from normal, whereas levels of intact human chorionic gonadotropin and the β subunit of human chorionic gonadotropin were both significantly reduced.

A practical advantage of the use of inhibin A as a screening marker is the very small change in average levels of inhibin A with increasing lengths of gestation between 15 and 18 weeks in women with unaffected pregnancies. Inaccuracies in estimating the length of gestation will therefore have a much smaller effect on the calculation of risk estimates than would be the case with a marker such as unconjugated estriol, whose levels change rapidly during gestation.[44] If the improved detection rate provided by inhibin A is confirmed in a larger series, then screening for inhibin A, alpha-fetoprotein, and the β subunit of human chorionic gonadotropin, in combination with maternal age, will be a very effective method for detecting Down's syndrome in the second trimester.

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COMBINING INHIBIN A WITH EXISTING SECOND-TRIMESTER MARKERS IN MATERNAL SERUM SCREENING FOR DOWN'S SYNDROME

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SUMMARY

To assess the value of inhibin A as an additional second-trimester maternal serum marker of Down's syndrome we studied 56 affected and 280 unaffected pregnancies matched for gestational age. The median level in the cases was 1·62 multiples of the gestation-specific median (MOM) in the controls, with 95 per cent confidence limits of 1·34–1·96. The distribution of inhibin levels in affected and unaffected pregnancies was approximately log Gaussian, with means about 1 standard deviation apart. This degree of separation was similar to that for human chorionic gonadotropin (hCG), free β-hCG, and unconjugated oestriol (uE_3), but about double that of alpha-fetoprotein (AFP) measured in the same samples. Inhibin was largely uncorrelated with AFP and uE_3, whereas the log correlation coefficient with hCG was 0·29 ($P=0·19$) for Down's syndrome and 0·41 ($P<0·0001$) for unaffected pregnancies; with free β-hCG, it was 0·18 ($P=0·38$) and 0·38 ($P<0·0001$), respectively. On the basis of these results and other pubished studies, we estimate that measuring inhibin A in addition to AFP and hCG or free β-hCG (with or without uE_3) will increase the detection rate for a fixed 5 per cent false-positive rate by about 7 per cent.

KEY WORDS: Down's syndrome; inhibin; maternal serum; second trimester

INTRODUCTION

Several studies have shown that maternal serum inhibin levels are raised on average in second-trimester pregnancies with Down's syndrome (Van Lith *et al.*, 1992; Spencer *et al.*, 1993; Cuckle *et al.*, 1994; Cuckle *et al.*, 1995; Canick *et al.*, 1994; Wallace *et al.*, 1996; Wald *et al.*, 1996; Aitken *et al.*, 1996). The intact inhibin molecule is a dimer with two possible forms (A and B) with a common α subunit and two distinct β subunits. The reported studies have used two types of assay, either an 'immunoreactive' assay which detects several inhibin species or one which is specific for inhibin A (α-β_A). One study compared both methods in 19 Down's syndrome pregnancies and found that the level of inhibin A was significantly higher than with the immunoreactive assay (Cuckle *et al.*, 1995). Whichever method is used, the potential of inhibin in Down's syndrome screening will ultimately turn on how much additional information it provides to existing markers. We carried out a study to assess this specifically for inhibin A.

MATERIALS AND METHODS

A series of maternal serum samples were selected for inhibin A measurement from a bank of samples which had been stored at -25°C or cooler. A total of 56 pregnancies affected by Down's syndrome were studied together with 280 unaffected control pregnancies. The gestational ages of the affected pregnancies in completed weeks were 13 (4 cases),

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14 (5), 15 (28), 16 (12), 17 (3), 18 (2), 19 (1), and 21 (1). Five controls were individually matched with each case for gestational age, which was based on ultrasound in all but three pregnancies. Serum samples were taken either routinely as part of the Down's syndrome screening programmes in Hull and Leeds or specifically for research purposes prior to an invasive diagnostic procedure or when there was a high Down's syndrome risk. The samples from cases comprised 52 taken for screening, three taken prior to prenatal diagnosis (two for ultrasound indications and one because of a family history of neural tube defects), and one because ultrasound indicated a high risk of Down's syndrome but prenatal diagnosis was refused.

All tests were carried out without knowledge of which sample was from an affected pregnancy and which a control. The inhibin results for 19 of the cases and their 95 controls have previously been reported (Cuckle *et al.*, 1995) using the inhibin A method described by Groome and O'Brien (1993). A modified assay was used for the remaining 37 cases and 185 controls. The standards were made from an in-house immunopurified preparation which was calibrated against the original recombinant human inhibin A preparation. Samples and standards (100 μl) were boiled for 3 min with 50 μl of 6 per cent (w/v) sodium dodecyl-sulphate (SDS), cooled, and oxidized with 10 per cent hydrogen peroxide (final v/v) for 30 min prior to assay. The assay methodology was thereafter as previously described except that detection was afforded by colour change of a simple alkaline phosphatase substrate (pNNP, Kierkegaard and Perry Laboratories, Maryland, U.S.A.), reading at 405 nm, rather than by the amplified detection system (Ampak, Dako, U.K.) used previously. The modified assay yielded inhibin concentrations about double those of the existing method, despite the similar gestations in the two sets of samples (medians of 15 weeks 4 and 3 days). To overcome this, we standardized inhibin values by dividing each one by the overall median concentration among controls in the appropriate set: 411 and 181 U/ml, respectively. The standardized values were then used to calculate normal medians for each gestational week and a regression analysis was performed, weighted for the number tested each week, so that multiples of the normal median (MOM) could be calculated for each day of gestation. A quadratic equation fitted best, giving $9{\cdot}44 - 0{\cdot}144\ \text{day} + 0{\cdot}000606\ \text{day}^2$. Finally, to adjust for maternal weight, the MOMs were divided by the expected value for the weight (in kg) from the $\log_{10}$ regression equation: 0·0173–0·000268 weight.

Each of the 336 samples had been previously tested for alpha-fetoprotein (AFP) and unconjugated oestriol (uE_3), with human chorionic gonadotropin (hCG) also measured in 154 samples and free β-hCG in the remainder. The tests on samples taken for screening were done prospectively and the research samples were tested in the next routine analytical batches after collection. Different commercial assay kits were used over the period of sample collection: immunoradiometric assay (IDS, Tyne and Wear, U.K.), radioimmunoassay (Amerlex-M, Johnson & Johnson Diagnostics Ltd, Amersham, U.K.; Biotecx, Texas, U.S.A.) or time-resolved fluorescent assay (Wallac Oy, Turku, Finland). The levels of all four analytes were expressed in MOM using gestation-specific medians for the appropriate period and laboratory, and adjusted for maternal weight.

Detection and false-positive rates were estimated by standard statistical modelling techniques (Royston and Thompson, 1992) for different combinations of inhibin A with AFP, uE_3, hCG, and free β-hCG. The model components are the maternal age distributions of affected and unaffected pregnancies and the frequency distributions of the markers. The age distributions were derived by applying published age-specific rates of Down's syndrome (Cuckle *et al.*, 1987) to the maternities in England and Wales for the period 1989–1993 (Office of Population Censuses and Surveys, 1991–1995). The existing marker distributions fit a multivariate log Gaussian distribution over a specified range. The fit of inhibin A alone and in combination with existing markers was tested using the Shapiro–Wilks test after excluding outliers exceeding 3 standard deviations from the mean. In the model, for the existing markers the multivariate distribution parameters were based on meta-analysis of published studies (Cuckle *et al.*, 1995). For inhibin, the parameters were first estimated from the current series as follows: the means from the medians, the standard deviations by dividing the tenth to 90th centile range by 2·563, and the correlation coefficients by first excluding outliers exceeding 3 standard deviations from the mean. These estimates were then entered into a meta-analysis with values from four other published series (Canick *et al.*, 1994; Wallace *et al.*, 1996; Wald *et al.*, 1996; Aitken *et al.*, 1996). For each

Table I—Inhibin and existing markers: tenth, median, and 90th centiles (MOM) in affected and unaffected pregnancies

	Down's syndrome			Unaffected		
Marker*	10th	Median	90th	10th	Median	90th
Inhibin A	0·83	1·62	3·60	0·56	1·00	1·80
AFP	0·49	0·85	1·32	0·68	1·00	1·61
uE_3	0·41	0·70	1·09	0·64	1·00	1·51
hCG	0·81	1·54	3·37	0·52	1·00	1·75
Free β-hCG	0·97	1·82	4·25	0·47	1·00	2·00

*Measured in all 56 cases and 280 controls except for hCG (26 cases, 128 controls) and free β-hCG (30 and 152, respectively).

parameter the average value was used, weighted for the number in each study.

RESULTS

There was a highly statistically significant elevation in inhibin A levels among Down's syndrome pregnancies ($P<0·0001$, Wilcoxon rank sum test). Table I shows the tenth, median, and 90th centiles in affected and unaffected pregnancies for inhibin and the other markers. The median inhibin level for Down's syndrome was 1·62 MOM with a 95 per cent confidence limit of 1·34–1·96 and was similar when using the existing and modified assays (1·64 and 1·58 MOM, respectively). There was no obvious tendency for the median to vary with gestation: at 13–14, 15–16, and 17–21 weeks, it was 1·72 (9 cases), 1·62 (40), and 1·48 (7) MOM, respectively.

The standard deviation of $\log_{10}$ inhibin was 0·25 and 0·20 for the cases and controls, respectively, so that the log median was elevated by 0·9 standard deviation (the average for affected and unaffected pregnancies). By comparison, the hCG and free β-hCG were raised by 0·8 and 1·1 standard deviations, respectively, with reductions in uE_3 and AFP of 1·0 and 0·5 standard deviation, respectively.

A correlation analysis between the logarithm of the marker levels showed that inhibin tended to have a positive association with AFP and the two species of hCG. The correlation coefficients for affected and unaffected pregnancies were respectively 0·08 ($P=0·57$) and 0·11 ($P=0·06$) for AFP, 0·29 ($P=0.19$) and 0·41 ($P<0·0001$) for hCG, and 0·18 ($P=0·38$) and 0·38 ($P<0·0001$) for free β-hCG. There was a tendency for a negative association with uE_3: $r=-0·08$ ($P=0·58$) and $r=-0·02$ ($P=0·78$) in affected and unaffected pregnancies, respectively.

Figure 1 is a probability plot for each inhibin A level. In such a diagram, a straight line indicates that the values follow a log Gaussian frequency distribution. Thus, the figure shows that for both affected and unaffected pregnancies there is little deviation from log Gaussian fit over a wide range of values. Formal testing confirmed this ($P=0·11$) for Down's syndrome and $P=0·07$ for unaffected pregnancies) and no significant deviations from multivariate fit were seen for inhibin together with AFP, uE_3, and free β-hCG ($P=0·61$ and 0·47 for affected and unaffected pregnancies, respectively); AFP, uE_3, and hCG ($P=0·11$ and 0·28); or AFP and free β-hCG ($P=0·13$ and 0·41).

Table II summarizes the inhibin parameters in this series and in three other publications together with the overall values from the meta-analysis. Based on this, the estimated detection rate for a fixed 5 per cent false-positive rate is 65·0 per cent for AFP, uE_3, and free β-hCG; 60·1 per cent for AFP, uE_3, and hCG; and 61·7 per cent for AFP and free β-hCG. The corresponding rates when inhibin is used as an additional marker are 71·6, 67·6 and 69·0 per cent, respectively. Therefore the use of inhibin is likely to increase detection by about 7 per cent.

DISCUSSION

In keeping with four other studies we found that the level of maternal serum inhibin A is raised on average in second-trimester pregnancies

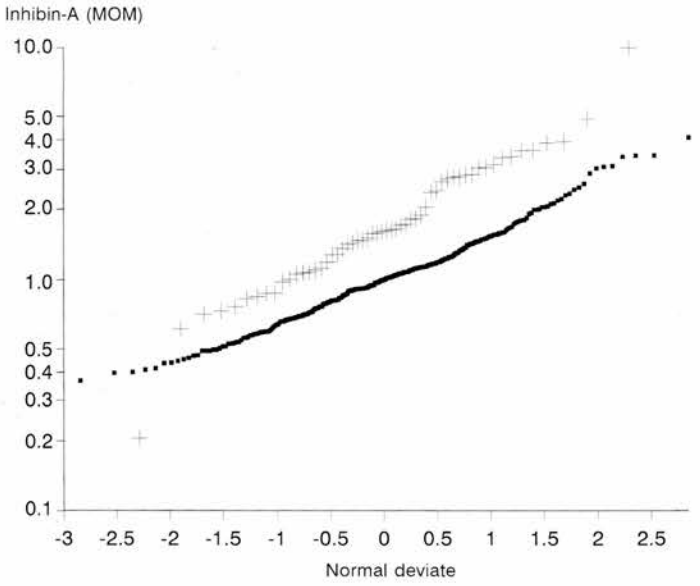

Fig. 1—Probability plot of inhibin A levels (in MOM) against the normal deviate of rank in 56 Down's syndrome (+) and 280 unaffected pregnancies (■)

Table II—Inhibin parameters (log_{10} MOM) for the multivariate Gaussian distributions in affected and unaffected pregnancies from four second-trimester case–control studies*

Study	Gestation (weeks)	Women		Mean	SD	*R* value AFP	uE_3	hCG	Free β
Canick *et al.*, 1994*	15–20	Case	20	0·27	0·26	0·28	0·20	0·57	—
		Control	100	0·00	0·23	0·24	0·02	0·36	—
Wallace *et al.*, 1996	15–17	Case	21	0·41	0·20	—	—	—	—
		Control	150	0·00	0·27	—	—	—	—
Wald *et al.*, 1996	13–22	Case	77	0·25	0·14	0·15	−0·15	0·36	0·50
		Control	385	0·00	0·22	0·08	0·02	0·19	0·23
Aitken *et al.*, 1996	15–18	Case	44	0·35	0·35	0·24	—	0·39	0·23
		Control	206	0·00	0·29	0·24	—	0·27	0·15
Present	13–21	Case	56†	0·21	0·25	0·08	−0·08	0·29	0·18
		Control	280	0·00	0·20	0·11	−0·02	0·41	0·38
Overall		Case	218	0·28‡	0·23	0·16	−0·08	0·38	0·36
		Control	1221	0·00	0·24	0·14	0·00	0·27	0·24

*And J. Canick, personal communication.
†Twenty-six cases had been tested for hCG and 30 for free β-hCG; similarly for 128 and 152 controls, respectively.
‡Equivalent to 1·91 MOM.

with Down's syndrome. Whilst the extent of elevation was slightly lower than that reported in the other studies, this is likely to be due to chance since the median hCG and free β-hCG levels in the cases were also lower than found elsewhere. The best estimate of the average inhibin A level in Down's syndrome can be derived from the studies combined. The geometric mean MOM value weighted for the number of affected pregnancies in each study is 1·91 MOM (95 per cent confidence interval 1·74–2·08).

Modifications were made to the original inhibin A assay method in order to improve analytical performance: a new standard preparation, a more cost-effective substrate detection system, and a pretreatment boiling step. A formal comparative study of these various changes has been performed and has confirmed that while absolute levels change, there remains a high degree of between method correlation except for the pretreatment step (Wallace, unpublished observations). Pretreatment allows the detection of inhibin A in haemolysed samples, which was not previously possible, and as a consequence leads to a poorer correlation with other methods. None the less, all the assay formats detect similar inhibin A species, which is not the case for the different immunoreactive inhibin assays. Moreover, the median MOM value was similar for Down's syndrome pregnancies tested with the original and modified assays.

The value in Down's syndrome screening of any marker is dependent on the amount of overlap between the distribution of values in affected and unaffected pregnancies. In our inhibin series, the degree of separation in terms of the number of standard deviations between the medians was similar to that of hCG, free β-hCG and uE_3, but about double that of AFP. The value of a marker in addition to established markers will depend on its correlation with them. For inhibin, the degree of correlation is not great and statistical modelling predicts that its use will increase the Down's syndrome detection rate by about 7 per cent. This estimate is necessarily provisional, due to the relatively small number of cases in the published series and the retrospective design of the studies. It is likely that in prospective screening the spread of inhibin results will be wider and the associations with the other markers smaller. None the less, it is reasonable to conclude that the use of inhibin A as an additional second-trimester Down's syndrome marker will be of material benefit.

Our study was confined to the second trimester of pregnancy. First-trimester inhibin levels have been reported for three series of pregnancies with Down's syndrome. One study used inhibin A and found that in 23 affected pregnancies the median value was 2·5 MOM (Wallace *et al.*, 1995). The others used immunoreactive inhibin assays which yielded medians of 1·3 MOM in 23 cases (van Lith *et al.*, 1994) and 1·2 MOM in 11 (Wallace *et al.*, 1994). A further study of inhibin A included samples over a 7- to 18-week range but tabulated results in three gestational groups (Aitken *et al.*, 1996). The median value was 0·98 MOM in 8 cases at 7–11 weeks, 2·60 MOM in 6 at 12–14 weeks, and 2·24 MOM in 44 at 15–18 weeks. Much more data will be required before any conclusions can be drawn about the discriminatory power of inhibin in relation to well-established first-trimester markers of Down's syndrome such as free β-hCG and pregnancy-associated plasma protein A (PAPP-A). Its role in first-trimester multimarker screening will depend on any correlations with these markers. Our preliminary results on 69 unaffected pregnancies indicate a high correlation with free β-hCG ($r=0·6$, $P<0·0001$) but not with PAPP-A ($r=0·1$).

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SECOND-TRIMESTER DIMERIC INHIBIN-A IN DOWN'S SYNDROME SCREENING

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SUMMARY

Initial studies of immunoreactive inhibin using a commercial assay have shown levels to be increased in three second-trimester series of maternal samples from Down's syndrome-affected pregnancies. This assay detected non-specifically all forms of circulating inhibin, dimeric and free alpha subunits, whether fully or partially processed. More recently, a new specific assay for dimeric inhibin-A has shown elevated results in both a first-trimester and a second-trimester series of cases. In order to assess the value of dimeric inhibin-A as a potential marker in the second trimester, we have analysed 157 Down's syndrome cases and used 367 unaffected cases across the gestational range 14–20 weeks to establish control medians and population parameters. In our series, the median MOM in Down's cases was 1·77, significantly higher than in the controls. At a 5 per cent false-positive rate, dimeric inhibin-A alone identified 37 per cent of cases. When used in conjunction with maternal age and other marker combinations, mathematical modelling showed detection rates rising from 48 per cent (inhibin-A plus age) to 61 per cent (inhibin-A, free beta hCG, age) and 68 per cent (inhibin-A, AFP, free beta hCG, age). Our data suggest that dimeric inhibin-A may have greater potential earlier in gestation when median levels at 14–16 weeks are 1·92 compared with 1·46 at 17–23 weeks. Dimeric inhibin-A may be a valuable addition to screening protocols, particularly in early gestations.

KEY WORDS: dimeric inhibin-A; second-trimester serum screening; Down's syndrome screening

INTRODUCTION

Inhibin is a member of the transforming growth factor beta (TGFβ) superfamily (Massague, 1990) and is characterized by its ability to suppress follicle-stimulating hormone (FSH) secretion (Ying, 1988). Fully processed inhibin is a dimer with a molecular mass of 32 kD, composed of an alpha subunit and one of two similar but distinguishable beta subunits. The resulting possible mature forms are dimeric inhibin-A (α-βA) and dimeric inhibin-B (α-βB), of which only dimeric inhibin-A is present in pregnancy sera (Wallace, 1995, unpublished observations; Illingworth *et al.*, 1996). In addition to these, however, a number of other partially processed and free α subunit forms may circulate peripherally (Schneyer *et al.*, 1990; Robertson *et al.*, 1995). Like placental gonadotropin-releasing hormone (Petraglia *et al.*, 1987), in pregnancy inhibin has been postulated to have a role in the feedback control of placental human chorionic gonadotrophin (hCG) secretion (Healy *et al.*, 1990).

In pregnancies affected by Down's syndrome, levels of hCG (either free beta hCG or total hCG) are increased in amniotic fluid (Spencer *et al.*, 1993a) and maternal serum (Spencer, 1993); as a result, free beta hCG is the most discriminating of the biochemical markers (Spencer, 1994a). In order to assess further the mechanisms involved in hCG regulation in cases of trisomy 21, we have previously investigated levels of immunoreactive inhibin using a commercial assay (Spencer *et al.*, 1993b). Using this same commercial assay, two other studies (Van Lith *et al.*, 1992; Cuckle *et al.*, 1994) have also demonstrated increased levels of immunoreactive inhibin in maternal serum samples from Down's syndrome-affected

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pregnancies. The commercial assay will detect non-specifically all forms of circulating inhibin, dimeric and free alpha subunits, whether fully or partially processed (Schneyer *et al.*, 1990). More recently, a new specific assay for dimeric inhibin-A has been developed (Groome and O'Brien, 1993; Groome *et al.*, 1994) and in studies of a first-trimester (Wallace *et al.*, 1995) and two second-trimester (Cuckle *et al.*, 1995; Wallace *et al.*, 1996) series of Down's cases, levels of inhibin-A have been shown to be elevated. In order to assess the value of dimeric inhibin-A as a potential marker of Down's syndrome in the second trimester, we have studied this marker in a large series of affected and unaffected cases.

MATERIALS AND METHODS

Patient population

The Down's study population consisted of women who presented through the NTD and Down's syndrome screening programme of the Romford centre. The serum collected from each woman was aliquoted and stored at $-20°C$ after being analysed in routine serum alpha-fetoprotein (AFP) and free beta hCG assays. The samples were collated on the basis of abnormal birth outcome or cytogenetic confirmation after mid-trimester amniocentesis as a result of an increased risk Down's syndrome screen report. A total of 157 singleton pregnancies associated with Down's syndrome were identified with maternal serum samples taken between 14 and 23 weeks' gestation and before amniocentesis. In order to provide a control population, 367 samples from unaffected pregnancies of the same gestational range were selected to establish the median value at each gestational week. Table I summarizes the study population. Both control and Down's syndrome samples had undergone only one previous freeze–thaw cycle.

Table I—Summary of the study population

	Controls	Down's cases
Maternal age (years)		
Mean	27·46	31·29
Range	15–43	17–47
Gestational age (weeks)		
Mean	17·19	16·29
Range	14–20	14–23
Storage time (months)		
Mean	30	40
Range	24–45	2–96

Analytical methods

AFP was measured using a polyethylene glycol-assisted radioimmunoassay (Spencer and Carpenter, 1985) and free beta hCG was measured using a highly specific solid-phase two-site immunoradiometric assay (ELSA-FbHCG; CIS (U.K.) Ltd, High Wycombe, Bucks, U.K.). The specificity, precision, and performance of this assay have been previously documented (Spencer, 1991a; Macri *et al.*, 1993). Dimeric inhibin-A was measured with a solid-phase sandwich microtitre plate ELISA (Groome and O'Brien, 1993; Groome *et al.*, 1994) with some minor modifications. Samples and standards were mixed with 6 per cent (w/v) sodium dodecyl sulphate, boiled for 3 min, cooled, and incubated at room temperature with 10 per cent (w/v) hydrogen peroxide for 30 min. The performance of this assay has been previously documented (Wallace *et al.*, 1995). The standard preparation used in this study was derived from immunopurified human follicular fluid, calibrated against previously used recombinant 32 kD human inhibin-A (Genentech, U.S.A.).

Statistical analysis

All results for each analyte were expressed in multiples of the median (MOM) for unaffected pregnancies at the same gestational age, derived from regressed weighted medians for each analyte when appropriate. Statistical analysis of the data was performed using Astute, a statistical software add-in for Microsoft Excel 5 (DDU Software, University of Leeds, U.K.). Calculation of the multivariate risk of Down's syndrome using combinations of various biochemical parameters, in addition to *a priori* maternal age risk (Cuckle *et al.*, 1987), was performed according to standard procedures as outlined by Reynolds and Penney (1990). To establish further the validity of various screening protocols applied to mass population screening, standard statistical modelling techniques (Royston and Thompson, 1992) were used. Population means and standard deviations were obtained in the manner used previously (Spencer *et al.*, 1992). Using the previously established

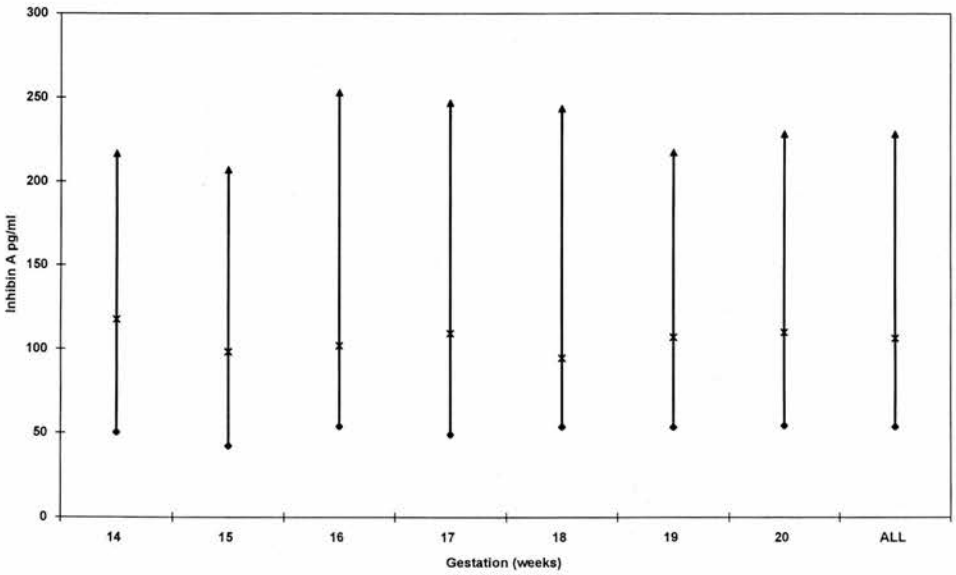

Fig. 1—Variation of dimeric inhibin-A with gestational age in the second trimester. × =median; ◆ =fifth centile; ▲ =95th centile

population parameters for AFP and free beta hCG (Spencer *et al.*, 1992) updated by the inclusion of data from a further 367 Down's syndrome cases and 49 858 unaffected cases, and the inhibin parameters established in this study, a series of random MOM values were selected from within the distributions of the affected and unaffected distributions. These values (without the use of truncation limits) were then used to calculate likelihood ratios and the expected Down's syndrome detection rate was calculated at a given false-positive rate (5 per cent) assuming the maternal age distribution of births in England and Wales (Office of Population Censuses and Surveys, 1991–1994). Repeated simulations through the model enabled confidence intervals to be established.

RESULTS

Figure 1 shows the median value (50th centile) and fifth and 95th centiles of the dimeric inhibin-A values at each gestational week. The results indicated almost no variation of the median value across the 14th to 20th week of pregnancy. When the levels at 14 weeks were compared consecutively with those in the 15th to 20th week using the Mann–Whitney *U*-test, no significant difference between medians at each week could be demonstrated ($P>0{\cdot}01$). Since gestational variation did not appear to be statistically significant, all subsequent MOM calculations were performed using the total population median (106·3 pg/ml).

Dimeric inhibin-A in both the affected and the unaffected cases was shown to fit a Gaussian distribution well after $\log_{10}$ transformation. The Kolmogorov–Smirnov *D* statistic for the Down's cases was 0·6288, and for the unaffected population 0·5831, neither significant at the 0·001 level. Figures 2 and 3 show the cumulative probability plots for these two populations. The $\log_{10}$ mean and standard deviation of the unaffected cases were 0·0117 and 0·1945, respectively, and for the Down's cases 0·2377 and 0·2284.

There was no evidence of any association of dimeric inhibin-A with maternal age. Correlation of $\log_{10}$ dimeric inhibin-A MOM with maternal age showed a correlation coefficient of $-0{\cdot}03$ in the unaffected population and $-0{\cdot}007$ in the Down's population.

Log_{10} dimeric inhibin-A showed a small but significant negative variation with maternal weight (log inhibin MOM $=0{\cdot}133-(0{\cdot}0019\times$ maternal weight in kg), $r=0{\cdot}1311$). This variation follows the same pattern seen with other biochemical markers and relates to an increase in the volume of distribution of the analyte as the maternal weight increases.

Dimeric inhibin-A in the unaffected population showed a median MOM of 1·00 [95 per cent

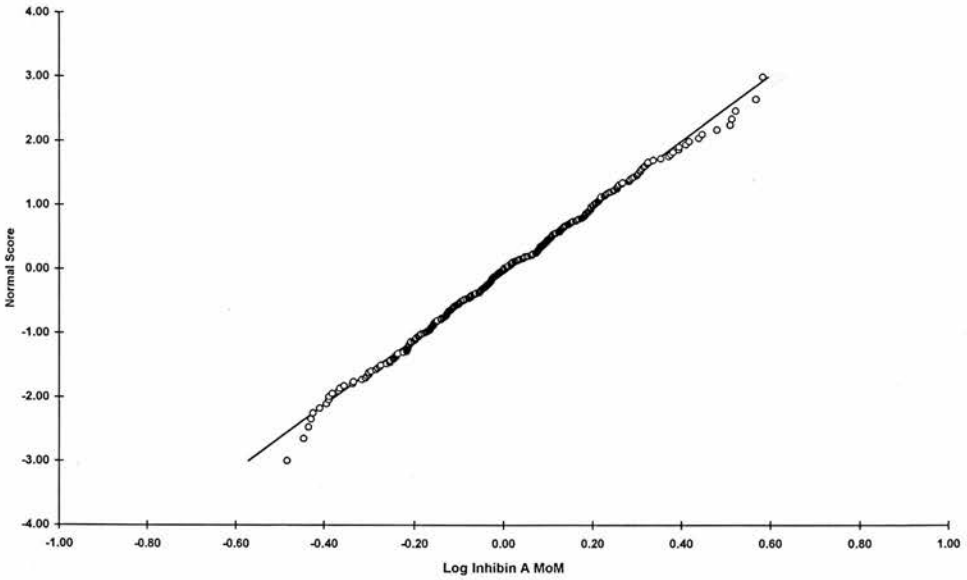

Fig. 2—Cumulative normal probability plot of $\log_{10}$ dimeric inhibin-A (MOM) in the unaffected cases

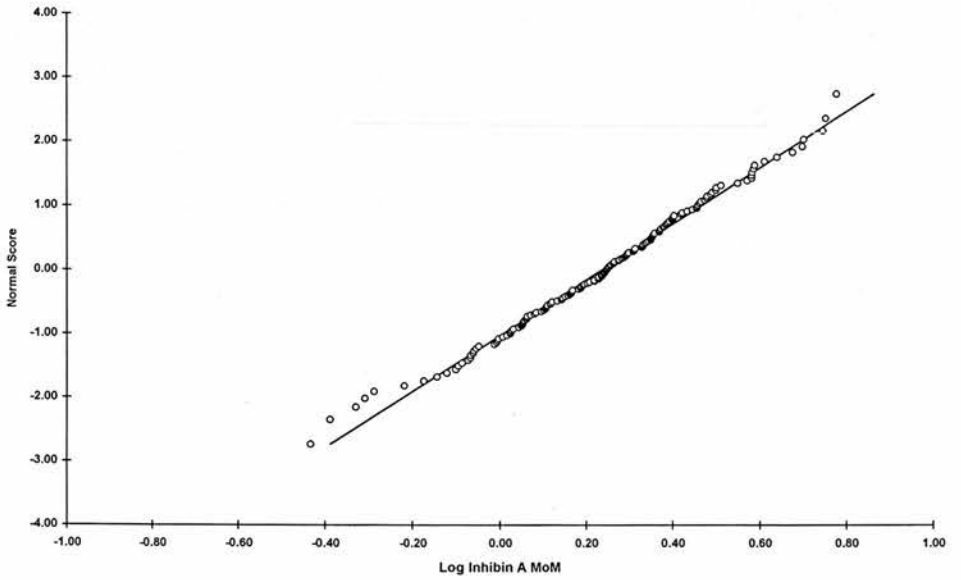

Fig. 3—Cumulative normal probability plot of $\log_{10}$ dimeric inhibin-A (MOM) in the Down's syndrome cases

confidence interval (CI) 0·94–1·07] with a tenth to 90th centile of 0·61–1·80 and a fifth to 95th centile of 0·50–2·11. In the Down's syndrome population, the median MOM was 1·77 (95 per cent CI 1·60–1·97), significantly greater ($P<0·0001$, Mann–Whitney test) than in the unaffected population. The tenth to 90th centile was 0·87–3·18 and the fifth to 95th centile was 0·75–3·95. Table II shows the distribution of affected and unaffected pregnancies with dimeric Inhibin-A at specific MOM cut-off values. Figure 4 shows the individual MOM values obtained in each of the Down's syndrome cases. At a fixed 5 per cent false-positive rate (2·11 MOM cut-off), dimeric inhibin-A alone

Table II—Distribution of affected and unaffected pregnancies at various dimeric inhibin-A MOM cut-off values

	Down's cases (n=157)		Unaffected cases (n=367)	
MOM	No.	%	No.	%
⩾0·50	153	98·1	350	94·9
⩾0·75	149	95·6	280	76·3
⩾1·00	134	85·3	186	50·6
⩾1·25	116	73·8	124	33·7
⩾1·50	98	62·2	78	21·1
⩾1·75	82	52·6	42	11·3
⩾2·00	63	39·8	26	6·9
⩾2·50	36	22·5	10	2·5
⩾3·00	22	13·5	6	1·4
⩾4·00	8	4·5	0	0

would have identified 59 of 157 cases (37·2 per cent) of Down's syndrome.

When the Down's syndrome cases were split into two groups based on those with a gestational age of 14–16 weeks (n=102) and those 17 weeks and older (n=55), there was an apparently higher median (1·92 MOM) amongst the cases at the earlier gestation compared with those at the later gestation (1·46 MOM). This was significant at the 0·0196 level using Mann–Whitney test statistics. The corresponding $\log_{10}$ mean and standard deviation (SD) were 0·262 and 0·238 at the earlier gestation and 0·195 and 0·205 at the later gestation.

When the dimeric inhibin-A MOM values were compared with previously measured AFP and free beta hCG values, a significant positive correlation was observed with free beta hCG in both the unaffected (r=0·2373) and the Down's syndrome group (r=0·3925). With AFP, the level of correlation was much smaller, with r equal to 0·1483 in the unaffected group and 0·0583 in the Down's group.

In the statistical modelling exercise, various combinations of the biochemical markers were used in conjunction with maternal age, and the population parameters for dimeric inhibin-A from the present study were combined with those

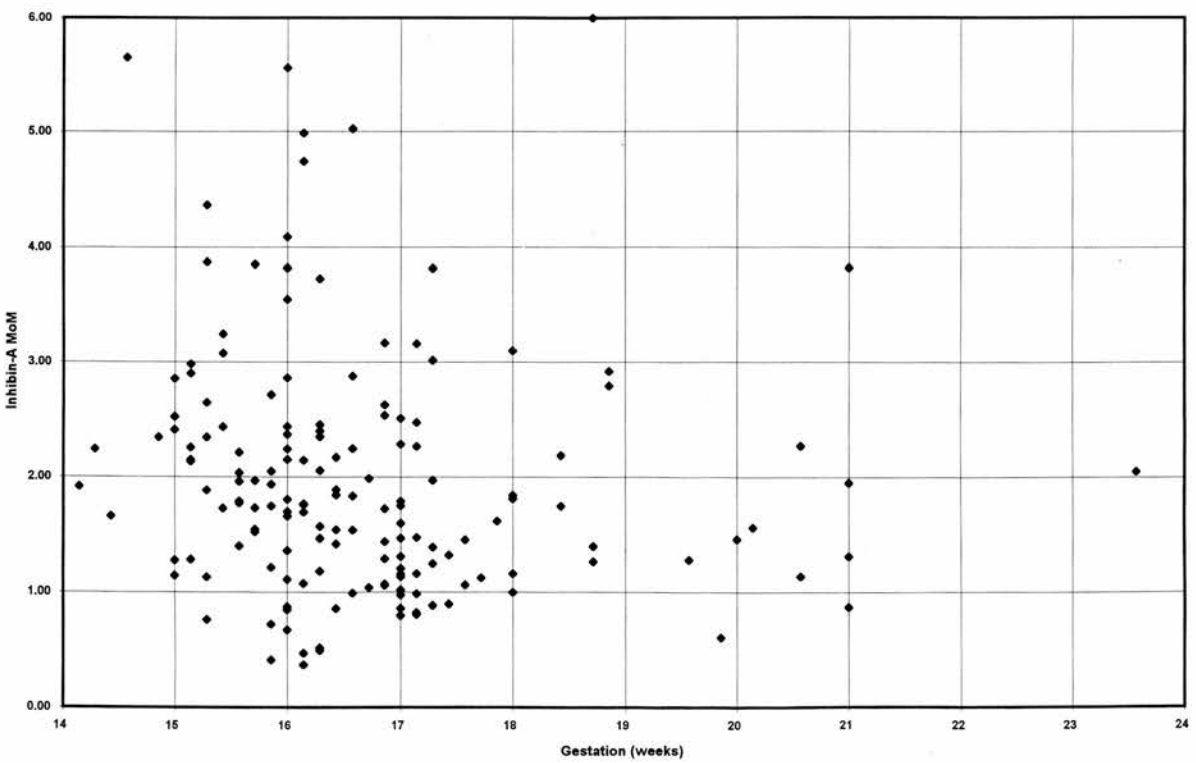

Fig. 4—Dimeric inhibin-A (MOM) in 157 cases of Down's syndrome

Table III—Expected Down's syndrome detection rates at a fixed 5 per cent false-positive rate for various combinations of biochemical markers and maternal age, using the England and Wales population model and repeated estimations through the simulation ($n=10$)

Test combination	Detection rate (%)	95% CI
Inhibin/age	47·8	45·9–49·7
Free beta/age	55·5	53·7–57·3
AFP/inhibin/age	55·9	54·0–57·8
Free beta/inhibin/age	60·5	58·8–62·2
AFP/free beta/age	64·5	62·8–66·2
AFP/free beta/inhibin/age	67·5	65·7–69·3

CI=confidence interval.

previously established for AFP and free beta hCG (Spencer *et al.*, 1992) corrected for population estimates in the extended series ($\log_{10}$ mean AFP controls=0·0, $\log_{10}$ SD AFP controls=0·1650, $\log_{10}$ mean AFP Down's= −0·1427, $\log_{10}$ SD AFP Down's=0·1868, $\log_{10}$ mean free beta controls=0·0, $\log_{10}$ SD free beta controls=0·2347, $\log_{10}$ mean free beta Down's=0·3848, $\log_{10}$ SD free beta Down's=0·2988, correlation controls=0·0172, correlation Down's= −0·0894, controls number=52 720, Down's number=457). The results of the simulation exercise are shown in Table III. The data indicated that in combination with maternal age, using dimeric inhibin-A alongside AFP and free beta hCG would increase Down's syndrome detection rates by no more than 3 per cent.

DISCUSSION

Our gestational median data are consistent with other published series which have similarly shown little or no variation of dimeric inhibin-A across the second-trimester 15- to 20-week period (Cuckle *et al.*, 1995; Canick *et al.*, 1994; Aitken *et al.*, 1996; Wald *et al.*, 1996). The overall median value from the control data set was 106·3 pg/ml; this is somewhat lower than the 181 pg/ml observed by Cuckle *et al.* (1995) and the 180 pg/ml observed by Aitken *et al.* (1996) using a similar assay calibrated directly with the recombinant inhibin-A. The median observed in the Wallace *et al.* (1996) study was higher at 237 pg/ml when using the same assay and recombinant inhibin-A calibrator as Cuckle *et al.* (1995) and Aitken *et al.* (1996). Wald *et al.* (1996), using the same assay as Cuckle *et al.* (1995), Wallace *et al.* (1996), and Aitken *et al.* (1996), but calibrated with a different recombinant inhibin-A preparation, found even higher median values at 346 pg/ml. The median observed by Canick *et al.* (1994), however, was significantly lower (67 pg/ml), although no details of the specific assay or calibration method were mentioned. The differences between the absolute inhibin-A levels reported in these various studies are a major cause for concern. Not only do they reflect differences in the various calibrator preparations in use, but they also reflect minor but important differences in assay formats which may be contributing to the absolute values of measured dimeric inhibin-A and, more importantly, to the levels of clinical discrimination of these various assay formats. Further studies are ongoing to evaluate the impact of changes in assay format and we await the production of an agreed international reference preparation for inhibin-A.

There was no evidence of any association of dimeric inhibin-A with maternal age, confirming previous studies in the first trimester (Wallace *et al.*, 1995) and the second trimester (Wald *et al.*, 1996).

The correlation with free beta hCG was not as high as had been observed previously (Spencer *et al.*, 1993b) with immunoreactive inhibin or as high as noted by Canick *et al.* (1994) when comparing intact hCG. The size of our correlation with free beta hCG was closely similar to that of Cuckle *et al.* (1995) when comparing intact hCG, which was much less than when the same samples were compared with immunoreactive inhibin (Cuckle *et al.*, 1994). Compared with the recent publication by Wald *et al.* (1996), the correlation with free beta hCG in the Down's group was lower in our study. Aitken *et al.* (1996), however, found a significantly lower level of correlation between inhibin-A and free beta hCG in both the controls and the Down's syndrome group, being almost half that observed in this study. Similarly, with intact hCG, these workers also found a much lower correlation than has been reported in other studies.

The median MOM value in our large Down's series was lower than that noted in a small preliminary series (Spencer *et al.*, 1993b) using a non-specific inhibin assay (1·77 vs. 3·60) but higher than noted by Cuckle *et al.* (1994) using the same non-specific assay (1·3) and closely similar to that initially published by Van Lith *et al.* (1992), again

using the same non-specific assay (1·9). Doubts have already been expressed about the contribution of the non-specific inhibin assay to the wide variation of results between these three studies (Cuckle *et al.*, 1994).

Cuckle *et al.* (1995), in re-analysing their samples using the more specific dimeric inhibin-A assay, found a small but significantly higher median MOM and their observed median of 1·6 MOM is of similar magnitude to that seen in our larger series. Canick *et al.* (1994), in a study of 20 Down's cases, found a similar median MOM of 1·88 and observed a similar distribution in both affected and unaffected populations ($\log_{10}$ SD unaffected 0·231, affected 0·261), although they reported a much higher degree of correlation with intact hCG in both affected and unaffected populations. The exact details of the dimeric inhibin-A assay used were not provided. Wallace *et al.* (1996), in a small study of 21 Down's cases, found a median of 2·6 MOM and observed a similar distribution in both affected and unaffected populations ($\log_{10}$ SD unaffected 0·27, affected 0·20). Ward *et al.* (1996), in their recent study, found a median of 1·79 MOM, which is closely similar to our study. However, our value of 1·77 MOM is lower than that reported by Aitken *et al.* (1996) in a study of 44 cases in the 15- to 18-week period. The $\log_{10}$ SD quoted by Wald *et al.* (1996) for inhibin-A in the unaffected group was 0·2188, similar to our figure of 0·1945, both being lower than the 0·2967 observed by Aitken *et al.* (1996). In the Down's syndrome group, Aitken *et al.* (1996) similarly observed a much wider distribution of inhibin-A levels ($\log_{10}$ SD=0·3521) and both control and affected data sets showed more than a 50 per cent increase in the width of the distribution, compared with our study. In the Wald *et al.* (1996) study, for the Down's group the authors quote a $\log_{10}$ SD of 0·1417 derived from an adjustment to the observed Down's data based on the differences between the variance and covariance for the matched control population and a newly collected set of unaffected samples. Examination of Table I in the Wald *et al.* paper shows a tenth to 90th centile in the Down's group of approximately 4·00 to 1·00; using the Wald *et al.* (1992) method of calculating SD shows that prior to this correction the SD approached 0·235, which is more consistent with all other published data. Wald *et al.*'s data also show considerable deviation in the tails of the unaffected and affected distributions, resulting in the authors suggesting data linearity only between 0·4 and 3·5 MOM. They suggest that in the Down's group they calculated the SD by only considering the population within the tenth to 70th centile; from their Table I, using the available data of the 7·8 centile (1·00 MOM) to the 71·8 centile (2·25 MOM), this should have produced an SD prior to their adjustment for variance and covariance differences between the older sample set and the fresh sample set of:

$$\text{SD} = (\log_{10} 2{\cdot}25 - \log_{10} 1{\cdot}00)/2 \times 0{\cdot}915 \quad \text{(the } z \text{ value at 64 per cent of the normal distribution)}$$
$$= 0{\cdot}1924$$

As has been pointed out previously (Spencer and Macri, 1994), this sample set was collected some 20 years ago (1973–1983) and has undergone repeated freezing and thawing, which may possibly affect the results. Our data clearly show linearity in the Down's samples from 0·6 MOM to 6·00 MOM and down to 0·3 MOM in the unaffected data set, with a better fit to linearity than that observed by Aitken *et al.* (1996) or Wald *et al.* (1996).

Clearly from our large study we can confirm that dimeric inhibin-A levels are increased in pregnancies affected by Down's syndrome, and when used alone (without maternal age), they can identify 37 per cent at a 5 per cent false-positive rate. In comparison with other markers, however, the extent of this elevation is less than with free beta hCG, which detects 46 per cent of cases, and similar to total hCG, which can detect 38 per cent of cases, but better than AFP or unconjugated oestriol (Spencer *et al.*, 1992). Our data are not as encouraging as the initial series from Wallace *et al.* (1996), who found a 62 per cent detection rate at a 5·3 per cent false-positive rate. Wald *et al.* (1996), however, only found a 24·7 per cent detection rate at a 5·5 per cent false-positive rate. The study by Aitken *et al.* (1996) did not report on the detection rate using the marker inhibin-A alone without maternal age, but from the standard deviation of the control and affected distributions it is possible to estimate from their data that at a 5 per cent false-positive rate (3·92 MOM) a detection rate of 25·5 per cent would have been achieved.

When combined together with maternal age and other biochemical marker combinations, mathematical simulation studies suggest that an increase in detection rate of little more than 3 per cent can be expected by adding dimeric inhibin-A to a simple two-analyte protocol of AFP and free beta hCG, which alone can be expected on average to give detection rates of 65 per cent and for which

prospective screening performance year on year seems to consistently show rates of 70–75 per cent to be achievable (Spencer, 1994b). If the population parameters for the inhibin-A $\log_{10}$ SD in the Down's group in the Wald *et al.* (1996) study are an underestimate, the net effect of this will have been to bias the results on detection efficiency towards showing an even greater benefit for the addition of inhibin-A. Our detection rates for the inclusion of inhibin-A are less optimistic than those of Aitken *et al.* (1996), who showed a projected 75 per cent detection rate using AFP/free beta hCG/inhibin-A/maternal age, being somewhat 22 per cent higher than that observed with only AFP/free beta hCG/maternal age. The poorer performance of the simple two-analyte protocol was some 12 per cent lower than detection rates observed in other studies and was noted by Aitken *et al.* as being a contributing factor to this performance differential. It is difficult to reconcile the wide observed standard deviations for inhibin-A in the Aitken *et al.* study with the projected 22 per cent increase in performance when inhibin-A is included.

On the basis of our predicted 3 per cent increase in detection rate, it is unlikely to be enough benefit to warrant the addition of a third and complex assay to such a simple two-analyte screening protocol as AFP and free beta hCG. The additional errors in the estimate of risk as more analytes are added to the risk algorithm have been well demonstrated both theoretically (Spencer, 1991b, 1994c) and practically (Seth *et al.*, 1995), and it is unlikely, as with the use of unconjugated oestriol (Spencer, 1994c), that such marginal benefits could be practically realized.

In the first trimester, dimeric inhibin-A using the more specific assay has been shown to be significantly elevated in one small study of 23 cases (Wallace *et al.*, 1995), contrary to two observations using assays measuring immunoreactive inhibin (Wallace *et al.*, 1994; Van Lith *et al.*, 1994). At this time, there is the same degree of correlation between free beta hCG and dimeric inhibin-A ($r=0{\cdot}6$ in Down's and 0·16 in controls) as in the second trimester but no correlation with pregnancy-associated plasma protein-A (PAPP-A) ($r=0{\cdot}052$ in Down's and 0·0005 in controls) (Wallace, 1995, unpublished observations). The recent study of Aitken *et al.* (1996), however, has shown no elevation of dimeric inhibin-A in a small study of eight cases in the 7- to 11-week period.

Dimeric inhibin-A MOM in Down's cases appears significantly higher in the earlier weeks of gestation in the second trimester (as also observed in the data of Aitken *et al.*, 1996); this mirrors that seen for free beta hCG (Macri *et al.*, 1990; Spencer *et al.*, 1993c), and may lead to higher detection rates in the 14- to 16-week period. It may be that dimeric inhibin-A will have an important role to play in the late first and early second trimester at a time when other potential first-trimester markers of Down's syndrome such as PAPP-A are rapidly losing their effectiveness (Aitken *et al.*, 1994).

Future work on dimeric inhibin-A must await clarification of how the different assay formats and the various calibrators contribute to the wide variation in published medians (67–346 pg/ml) and whether these differences also contribute to variation in the Down's syndrome detection rate.

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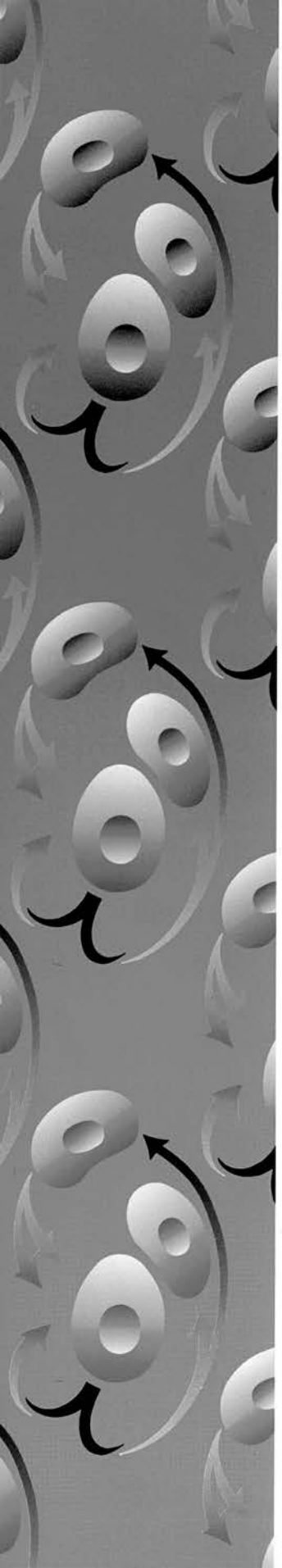

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Amniotic fluid inhibin-A in chromosomally normal and Down's syndrome pregnancies

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Abstract

Recently, inhibin-A has been shown to be a useful new prenatal marker of Down's syndrome, significantly increasing detection rates. While the placenta is believed to be the major source of inhibin in pregnancy, there are actually very limited data available on specific inhibin dimers in pregnancy. Using a sensitive and specific ELISA we have measured the inhibin-A content of amniotic fluid (AF) to investigate further the biology of inhibin-A in chromosomally normal and abnormal pregnancies. AF from 51 Down's syndrome and 161 chromosomally normal pregnancies between 16 and 19 weeks of gestation were analysed, blinded as to whether the sample was from a Down's syndrome or normal pregnancy. There were no sex differences in inhibin-A content in either the control or Down's syndrome pregnancies. The median (10th–90th percentiles) inhibin-A level in the control pregnancies increased from 339·6 (175·2–649·1) pg/ml at 16 weeks to 592·9 (256·4–1027·3) pg/ml at 19 weeks of gestation. The median (95% confidence interval) inhibin-A in the Down's syndrome pregnancies, expressed as multiples of the median (MoM) to correct for gestation, was 0·77 (0·68–0·89) MoM, significantly lower than the controls ($P<0·001$, Mann–Whitney U test).

We believe that these data are compatible with more than one source of inhibin-A in pregnancy and suggest that the fetal membranes may be contributing significantly to AF inhibin-A content. Further, our data would suggest that the endocrine function of the placenta and the other inhibin source(s) are differentially regulated.

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Introduction

Inhibins are dimeric glycoproteins characterised by their ability to suppress follicle-stimulating hormone secretion from the anterior pituitary gland (Ying 1988). Two fully processed forms of inhibin exist – inhibin-A (an α-β_A dimer) and inhibin-B (an α-β_B dimer) and while the ovary secretes both of these forms during the normal menstrual cycle (Groome *et al.* 1994, 1996), the principal source of inhibin in pregnancy, the placenta, only secretes inhibin-A (Muttukrishna *et al.* 1995, Illingworth *et al.* 1996, Wallace *et al.* 1996*a*).

It has recently been shown that, compared with normal pregnancy, maternal serum (MS) inhibin-A levels are elevated in Down's syndrome pregnancies, in both the first (Wallace *et al.* 1995) and second (Cuckle *et al.* 1995, Aitken *et al.* 1996*a*, Wallace *et al.* 1996*b*) trimesters. Further, when combined with maternal age and other MS markers of Down's syndrome, inhibin-A will add a further 10–20% detection to current established marker combinations (Aitken *et al.* 1996*a*, Wald *et al.* 1996). Inhibin-A would thus appear to be a promising new prenatal marker of Down's syndrome.

To explore the biology of inhibin-A secretion in pregnancy further we have studied inhibin-A levels in amniotic fluid (AF) in chromosomally normal and Down's syndrome pregnancies.

Materials and Methods

Fifty-one Down's syndrome pregnancies, from which AF had been collected prospectively as part of the West of Scotland prenatal diagnosis service, were identified from records and an aliquot of fluid retrieved from storage at − 20 °C. Of these 51 pregnancies, 11 (21·6%) had an amniocentesis performed because of maternal age and 40 (78·4%) because of an increased risk by α-fetoprotein (AFP)/age or AFP/human chorionic gonadotrophin (hCG)/age screening. Similarly, 161 chromosomally normal control pregnancies on which an amniocentesis had been performed were identified from records and an aliquot of fluid retrieved. Sixty-eight (42·2%) of these amniocenteses had been performed for maternal age, 82 (50·9%) for a positive MS screening result, one (0·6%) for an isolated elevated MS AFP and 10 (6·2%) for

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Table 1 Inhibin-A levels (pg/ml) in AF from 161 chromosomally normal and 51 Down's syndrome pregnancies between 16 and 19 weeks of gestation

	Controls		Down's syndrome	
	No. of samples	Median inhibin-A (10th–90th percentiles)	No. of samples	Median inhibin-A (10th–90th percentiles)
Week of gestation				
16	45	339·6 (175·2–649·1)	11	158·9 (106·4–387·6)
17	46	485·7 (221·5–829·7)	16	314·7 (99·2–558·1)
18	46	362·2 (138·4–893·0)	21	349·3 (170·6–560·8)
19	24	592·9 (256·4–1027·3)	3	430·1

other miscellaneous reasons (e.g. past history, maternal anxiety).

Inhibin-A was measured in each sample using an ELISA specific for inhibin-A (Groome & O'Brien 1993), blinded as to whether the sample was from a Down's syndrome or a normal pregnancy. The original assay method was modified and validated for AF as previously reported (Wallace *et al.* 1996*a*).

Statistical analyses were performed using Statview 4·1 (Abacus Inc, Berkeley, CA, USA) and SPSS for Windows (SPSS Inc, Chicago, IL, USA). AF inhibin-A levels increase significantly and linearly across the gestational window of 14 to 20 weeks (Wallace *et al.* 1996*a*). The inhibin-A levels in both the Down's syndrome and control pregnancies were therefore expressed as multiples of the normal median (MoM), allowing a comparison between the two groups. MoMs, for both the Down's syndrome and control samples, were calculated from the regressed median of the controls at the appropriate gestation. The use of regressed medians corrects for the artefactual variation that arises secondary to the small sample size and so affords more accurate MoM estimates for both the control and Down's syndrome samples than using non-regressed medians. The regressed medians were calculated using the weighted regression equation: $\text{median}=e^{(8\cdot0649-35\cdot045/\text{gestation})}$.

Results

Seventy-eight (48·4%) of the 161 control AF samples were from pregnancies with a female fetus, 82 (50·9%) were from a pregnancy with a male fetus and one (0·6%) was unknown. Of the Down's syndrome samples, 18 (37·3%) and 32 (62·7%) were from pregnancies with a female and male fetus respectively. There were no significant differences in inhibin-A levels between the sexes for either controls or Down's syndrome cases (data not shown) and so the data were subsequently analysed by group, combining data for both sexes therein. Table 1 displays the number of control samples at each gestation and the median, 10th and 90th percentiles for inhibin-A in these samples. The median inhibin-A level at 19 weeks of gestation was significantly higher than at the other periods of gestation ($P<0·01$, Mann–Whitney U test). Figure 1 shows the regressed medians, 10th and 90th percentiles across the gestations.

Table 1 shows the median inhibin-A levels in the 51 Down's syndrome samples by week of gestation. Expressed as MoMs, the median (95% confidence interval) inhibin-A level for the Down's syndrome pregnancies was 0·77 (0·68–0·89) MoM (Fig. 2). The level of inhibin-A in AF from the Down's syndrome pregnancies was significantly lower than in the controls ($P<0·0002$, Mann–Whitney U test).

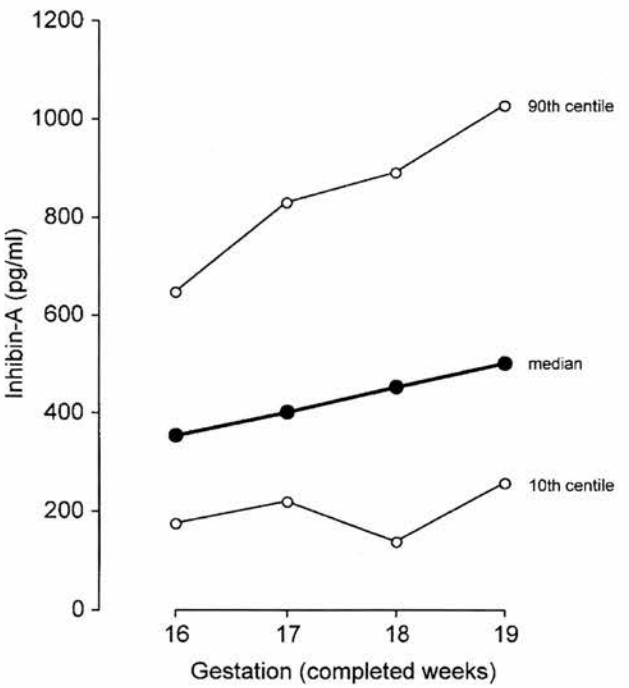

Figure 1 AF inhibin-A levels (regressed medians, 10th and 90th centiles) in 161 chromosomally normal pregnancies at 16–19 weeks of gestation.

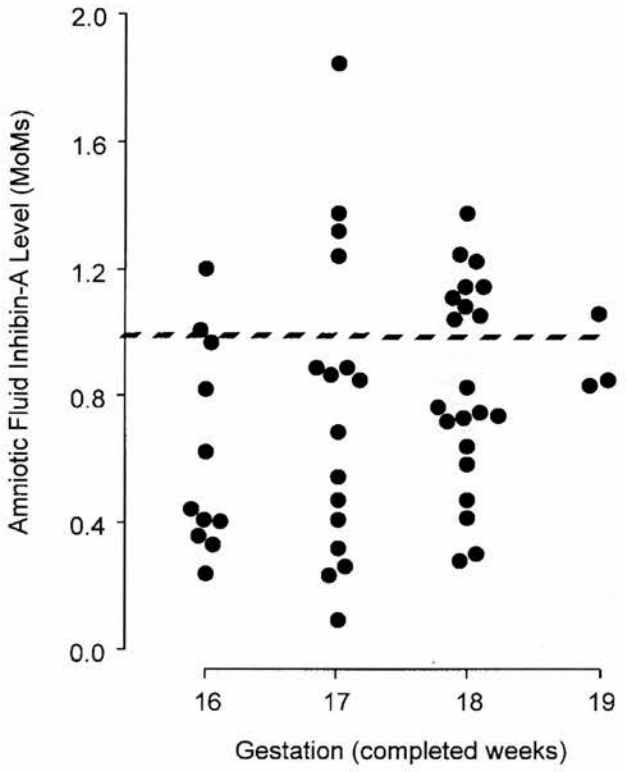

Figure 2 AF inhibin-A levels, expressed as multiples of the regressed normal median (MoMs), in 51 Down's syndrome pregnancies.

Discussion

The ontogeny of inhibin-A secretion in early pregnancy is similar to that of hCG with levels peaking at approximately 9–10 weeks of gestation (Aitken *et al.* 1996*a*, Illingworth *et al.* 1996). Levels then decline to a nadir at 17 weeks (Muttukrishna *et al.* 1995, Aitken *et al.* 1996*a*, Wallace *et al.* 1996*a*), rising thereafter such that the highest levels are achieved by term (Muttukrishna *et al.* 1995). It is generally accepted that the principal source of inhibin in pregnancy is the placenta (reviewed by Qu & Thomas 1995). Although the control of placental inhibin secretion has yet to be fully elucidated, hCG stimulates inhibin secretion from *in vitro* trophoblast cell cultures and, reciprocally, inhibin suppresses hCG secretion (Petraglia *et al.* 1987).

MS hCG is elevated in Down's syndrome (Bogart *et al.* 1987, Chard & Iles 1994), reflecting increased production of both hCG subunits by the placenta (Eldar-Geva *et al.* 1995). Considering the *in vitro* and *in vivo* relationships of hCG with inhibin it is not unexpected that MS immunoreactive inhibin (van Lith *et al.* 1992, Spencer *et al.* 1993), and more specifically inhibin-A (Cuckle *et al.* 1995, Wallace *et al.* 1995, 1996*b*, Aitken *et al.* 1996*a*, Wald *et al.* 1996) levels are also higher than normal in pregnancies complicated by Down's syndrome. However, despite these apparent similarities there are important differences between the secretion of hCG and inhibin in both normal and Down's syndrome pregnancies. In normal pregnancy the intact hCG concentration is much lower in AF than in MS (Kletsky *et al.* 1985) while inhibin-A concentrations in AF are significantly higher than in MS (Wallace *et al.* 1996*a*). In Down's syndrome pregnancies, while both intact hCG and free βhCG levels in AF are higher than in AF from normal pregnancies (Cuckle *et al.* 1991, Aitken *et al.* 1996*b*), comparable with the differences reported in serum (Bogart *et al.* 1987, Chard & Iles 1994), we have shown that AF levels of inhibin-A in Down's syndrome pregnancies are significantly lower than in normal pregnancy AF, the reverse of that which is observed in MS for this protein. There are a number of possible explanations for this unexpected finding for inhibin-A.

The lower levels of AF inhibin-A observed in Down's syndrome compared with normal pregnancies may relate to changes in either the route of secretion of inhibin-A by the placenta, in preference for MS, or in the rate of clearance of inhibin-A from the amniotic cavity. While MS levels of several proteins and steroids are deranged in Down's syndrome, the AF levels for each of these are similarly deranged (Cuckle *et al.* 1985, 1991), indicative of neither an altered route of secretion nor selective changes in clearance from AF.

Dimeric inhibin-A in AF may dissociate into separate subunits more rapidly in Down's syndrome relative to normal pregnancy. Levels of free βhCG and free αhCG subunits are much higher in AF than in MS while intact hCG levels are lower (Ozturk *et al.* 1988, Aitken *et al.* 1996*b*). The increased concentration of free hCG subunits in AF compared with MS could be accounted for by an increased breakdown of intact hCG in AF but it is thought more likely that the chorionic trophoblast preferentially secretes free subunits into AF and the placental trophoblast secretes intact hCG into MS (Ozturk *et al.* 1988). In the first trimester of pregnancy the major source of hCG in extra-embryonic coelom, and thereafter AF, is probably chorionic, as opposed to placental, trophoblast (Iles *et al.* 1992, Chard *et al.* 1995). It is therefore possible that in the second trimester the differences between MS and AF in intact and free subunit hCG levels may represent different sources.

Thus, the source of AF inhibin-A may be different from that of MS. Indeed, it has been previously suggested that the placenta may secrete inhibin-A primarily into MS while the chorion is probably the main source of inhibin-A in AF (Wallace *et al.* 1996*a*). However, if this were so then our data would indicate that the abnormality in Down's syndrome underlying the elevated MS inhibin-A levels would have to be differentially expressed in the placental and chorionic trophoblast. This would be quite unlike hCG (either intact or free βhCG).

In summary, we have demonstrated that the AF inhibin-A concentration in Down's syndrome is significantly lower than in normal pregnancy. The precise explanation for this unexpected finding currently remains obscure but our data are consistent with more than one significant source of inhibin-A in mid-pregnancy and with differential control of these sources. Studies exploring changes in mRNA for the three inhibin subunits in the placenta and fetal membranes in normal and Down's syndrome pregnancies would afford some clarification of this possibility and would certainly now be worthwhile.

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Dimeric Inhibins in Amniotic Fluid, Maternal Serum, and Fetal Serum in Human Pregnancy*

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ABSTRACT

Using new specific and sensitive enzyme-linked immunosorbent assays for inhibin A and inhibin B, we measured these proteins in amniotic fluid (AF), maternal serum (MS), and umbilical cord serum in normal pregnancies.

Inhibin A levels in AF rose from a median (10–90th percentile) level of 615 (158.2–1124.6) pg/mL at 14 weeks to 1336.0 (489.4–2084.1) pg/mL at 20 weeks, and inhibin B rose from 216.6 (67.4–554.6) to 1078.2 (439.3–2482.2) pg/mL over the same period. In MS, inhibin A levels fell from a median (10–90th percentile) level of 177.5 (101.4–290.7) pg/mL at 10 weeks to a nadir of 111.9 (59.5–200.3) pg/mL at 17 weeks, rising again to 180.3 (74.1–327.2) pg/mL at 20 weeks. No inhibin B was detectable in MS. In 47 pairs of matched samples (14–16 weeks gestation) there was no correlation of inhibin A levels in AF with those in MS ($r = 0.19$; $P > 0.05$). In 45 term umbilical cord serum samples, no dimeric inhibin was detectable in serum from female babies, but inhibin B was detectable in male sera; the median (10–90th percentile) concentration was 167.4 (111.2–224.8) pg/mL.

These data suggest that for the gestation periods studied, although the placenta secretes inhibin A, another source, probably the fetal membranes, secretes both inhibin A and inhibin B. Further, the presence of inhibin B in male fetuses is consistent with a testicular origin, suggesting that inhibin B may be important in the development of the fetal hypothalamo-pituitary-testicular axis. (*J Clin Endocrinol Metab* **82:** 218–222, 1997)

INHIBINS ARE glycoproteins that belong to the transforming growth factor-β superfamily (1) and are characterized by their ability to suppress FSH secretion (2). They are composed of an α-subunit and one of two β-subunits, β_A or β_B, giving rise to two mature 32-kDa inhibins: inhibin A (α-β_A) and inhibin B (α-β_B) (2). Although inhibins were originally identified from gonadal tissue, messenger ribonucleic acids (mRNAs) for the inhibin subunits are expressed in many nongonadal sites (3), including the placenta (4). Indeed, very high circulating levels of inhibin have been reported during pregnancy, significantly higher than those in nonpregnant subjects (5–8), which then rapidly decline after delivery (6, 8, 9). Current evidence derived from clinical studies (4–11), from the localization of inhibin subunit mRNA (4, 12) and proteins (4, 13), and from trophoblast cell culture experiments (12) suggests that the placenta is probably the principal source of inhibin during pregnancy (14).

Until recently, the available assays for inhibin were unable to differentiate between dimeric forms and partially processed free α-subunits or between dimers (15–17). Thus, our current understanding of inhibins in pregnancy is largely based upon these nondiscriminatory immunoreative inhibin assays (14). However, the development of sensitive and specific enzyme-linked immunosorbent assays (ELISAs) for inhibin A (18) and inhibin B (19) and their subsequent application have offered novel and important insights into inhibin biology in chromosomally abnormal pregnancies (20–22). To gain further insight into the biology of inhibin in normal pregnancy, therefore, we measured inhibin A and inhibin B in different pregnancy compartments.

Materials and Methods

Samples

Amniotic fluid (AF). Aliquots of AF were obtained from the regional cytogenetics laboratory in Edinburgh, Scotland. These had been collected prospectively as part of a clinical amniocentesis service. AF was separated from fetal epithelial squamous cells by centrifugation at 250 × *g* within 24 h of collection, and an aliquot of each sample was stored specifically for this study at −20 C until assay. The karyotype from each sample was reported subsequently.

Maternal serum (MS). Blood samples were collected prospectively as part of the West of Scotland Down's Syndrome and Neural Tube Defects Prenatal Screening Program and from an ongoing first trimester study of serum markers. Each sample was centrifuged, and the serum was separated within 3 days of collection and stored at −20 C. Information from early pregnancy karyotyping, where performed, and birth records, where karyotyping is not performed, is routinely used to identify chromosomally abnormal babies. Through the exclusion of these, sera from chromosomally normal pregnancies were identified and retrieved from storage. To gain sex details from this normal group, a cohort of case records was retrieved and analyzed.

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Matched MS and AF. In 47 women venepuncture was performed, with informed consent, immediately before amniocentesis (14–16 weeks gestation). The blood was centrifuged, and the serum was separated on the day of collection and stored at −20 C until assay. The AF was processed as detailed above. In each case, a normal karyotype was subsequently reported.

Umbilical cord serum. In 45 normal pregnancies, at term (37–41 weeks) and in normal spontaneous labor, umbilical blood (arterial and venous) was collected after consent was obtained. After the delivery of each baby, the cord was double clamped, and blood was taken after delivery of the placenta. Blood was centrifuged on the day of collection, and serum was separated and stored at −20 C until assay.

For each sample, AF, MS, or umbilical cord serum, the time of gestation at sampling, in completed weeks, was calculated from certain menstrual dates or from an early pregnancy ultrasound scan. Ethical approval was granted by the Lothian research ethics committee.

Assays

Inhibin A. Inhibin A was measured using a two-site ELISA (18) that has been previously validated for human serum (23) but with some modifications. Before assay, samples (and standards) underwent two preparatory steps. Each was mixed with 2% (final wt/vol) SDS and heated in a water bath at 100 C for 3 min. After cooling, each sample or standard was mixed with 1% (final wt/vol) hydrogen peroxide, incubated at room temperature for 30 min, and then diluted 1:1 in assay diluent (0.1 mol/L Tris-HCl, 0.15 mol/L NaCl with 5% Triton X-100, 10% BSA, and 5% normal mouse serum, pH 7.5). The assay uses an immobilized anti-βA-inhibin subunit monoclonal antibody (E4) as a capture antibody, covalently coupled to hydrazide microplates (Avidplate-HZ, UniSyn Technologies, Tustin, CA). The Fab fraction of a mouse anti-α-inhibin subunit monoclonal antibody (R1) is used as a second antibody diluted in assay diluent. This is conjugated to alkaline phosphatase, allowing detection by the addition of an alkaline phosphatase substrate, *p*-nitrophenylphosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Inhibin, immunopurified from follicular fluid and calibrated against recombinant 32-kDa human inhibin A (Genentech, South San Francisco, CA), was used as a standard preparation. Plates were read at 405 nm in a microplate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA) using dedicated software (Softmax, Molecular Devices Corp.). Results are expressed as picograms per mL, with an assay sensitivity of 23 pg/mL. The intra- and interplate coefficients of variation were 4.3% and 5.6%, respectively. The cross-reactivity of this assay with activin A, activin B, follistatin, purified human pro-αC, and inhibin B is less than 0.1%. The recovery of recombinant human inhibin A spiked into AF was quantitative (mean ± SEM, 108 ± 13%; n = 10). Serial dilution of AF samples gave dose responses parallel to that of the immunopurified standard (Fig. 1).

Inhibin B. Inhibin B was measured using a similar two-site ELISA as previously described (19). A monoclonal antibody (C5) raised against the human inhibin β_B-subunit was used as a capture antibody, biotinylated, and immobilized on streptavidin-coated microplates (Life Sciences International, Basingstoke, UK). The same second antibody (R1) as that used in the inhibin A assay was employed. Samples and standards were also pretreated as detailed above, but in this assay the signal was detected using an alkaline phosphatase amplification kit (Life Technologies, Paisley, UK). The same immunopurified inhibin preparation as that detailed above was used as a standard but calibrated against recombinant human inhibin B, with results expressed in picograms per mL. Plates were read at 490 nm using the same apparatus and software as those described for the inhibin A assay. The assay detection limit was less than 5 pg/mL. Activin A, activin B, follistatin, and purified human pro-αC had less than 0.1% cross-reaction, whereas recombinant inhibin A had 0.5% cross-reaction. The intra- and interplate coefficients of variation were 7.6% and 8.1%, respectively. Recovery of recombinant human inhibin A spiked into AF was quantitative (mean ± SEM, 88 ± 11%; n = 8). Serial dilution of AF gave a dose response parallel to that of the immunopurified standard (Fig. 1).

Statistical analyses were performed using Statview 4.1 (Abacus, Berkeley, CA) and SPSS for Windows (SPSS, Chicago, IL). Regressed medians were calculated to compare the ontogeny of the inhibins in each compartment, and multiples of the median (MoM) were used to correct for gestational changes, allowing group comparisons across gestations.

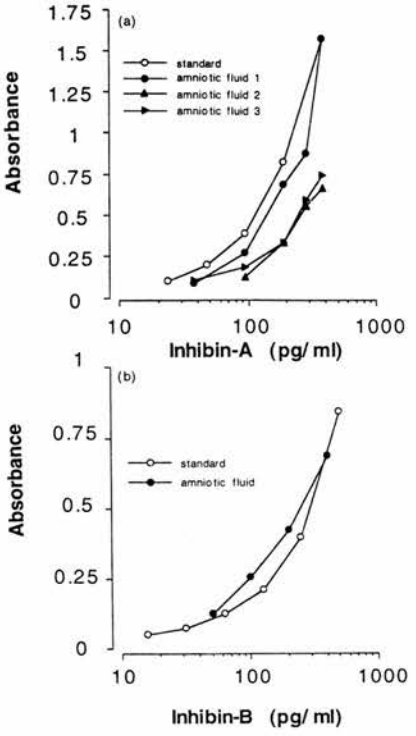

FIG. 1. Dose-response relationships for immunopurified inhibin standards with serially diluted amniotic fluid samples in the inhibin A (a) and inhibin B (b) ELISAs.

The regression equations calculated were: AF inhibin A: median = $e^{(11.3209-84.616/\text{gestation})}$; AF inhibin B: median = $e^{(9.3254-41.656/\text{gestation})}$; MS inhibin A: median = $-0.0129006x^5 + 0.988003x^4 - 29.6031x^3 + 434.528x^2 - 3136.00x + 9078.01$ (where x is weeks gestation).

Results

AF

AF samples from 618 consecutive amniocenteses were available for measurement of inhibin A content. In 15 (2.4%), karyotyping was abnormal, and these were excluded from this study. Of the 603 AF samples analyzed, 388 (64.3%) were unselected with regard to placental function [tests were performed for reasons of maternal age (n = 350), maternal anxiety (n = 19), or past history (n = 19)] and were grouped together (group A). The remaining 215 (35.7%) amniocenteses (group B) had been performed after an abnormal ultrasound scan (n = 15) or after positive MS screening, based on maternal age and MS levels of intact hCG and alphafetoprotein, had indicated an increased risk of fetal trisomy 21 (n = 200).

Inhibin A levels were analyzed for groups A and B separately by completed week of gestation and by fetal sex. There were no significant differences in inhibin A levels between the two groups ($P > 0.05$, by Mann-Whitney U test; data not shown) or between sexes in either group A or B ($P > 0.05$, by Mann-Whitney U test; data not shown). Data from all AF samples were, therefore, combined regardless of in-

dication for sampling or fetal sex and analyzed by gestational age. Table 1 details the median and 10th and 90th percentiles for inhibin A in AF in these 603 pregnancies. Levels increased steadily across the gestational window of 14–20 weeks, as evidenced by the regressed medians (Fig. 2).

Inhibin B assays were performed on 189 of the 603 AF samples. Where possible (weeks 14–18 inclusive), all samples were from group A, with 15 samples for each sex at each gestation. At 19 and 20 weeks, however, there were insufficient numbers in group A alone, and 7 samples at each gestation (1 female and 6 males at 19 weeks and 3 females and 4 males at 20 weeks) were derived from group B. There were no significant differences in inhibin B levels between the 23 AF samples from group A and the 7 from group B at 19 weeks gestation or between the sexes at any time of gestation ($P > 0.05$, by Mann-Whitney U test; data not shown). The data were, therefore, grouped by gestation, combining both sexes within each group. Table 2 details the median and 10th and 90th percentiles for inhibin B in these 189 pregnancies. Levels increased steadily across the gestational window, as shown in Fig. 2.

There was significantly more inhibin A than inhibin B at 14–16 weeks gestation ($P < 0.001$, by Mann-Whitney U test), but because the levels of inhibin B rose more rapidly, this became nonsignificant at 17 weeks (Fig. 2). There was a significant, but weak, association between the levels of inhibin A and B in the 189 amniotic fluid samples ($r = 0.42$; $P = 0.0001$).

MS

Sera from 807 chromosomally normal singleton pregnancies from 10–20 completed weeks of pregnancy were analyzed for inhibin A and inhibin B. Table 3 details the distribution of these sera by gestation and the median (10th and 90th percentiles) inhibin A levels. Levels fell significantly from 10 weeks to a nadir at 17 weeks ($P < 0.0001$, by Mann-Whitney U test), rising again to 20 weeks ($P < 0.0001$, by Mann-Whitney U test; Fig. 2).

Sexing information was available from the case records of 165 of the 807 pregnancies, 91 males and 74 females. When expressed as multiples of the median (MoM), correcting for gestation, there were no sex differences (1.02 MoM *vs.* 1.04 MoM for males and females, respectively; $P = 0.88$, by Mann-Whitney U test).

Inhibin B was undetectable in MS.

Matched MS and AF

Inhibin A was measured in matched maternal sera, and AF samples were collected from 47 women at 14–16 weeks gestation. The median (10–90th percentiles) inhibin A levels in MS and AF were 123.7 (79.0–196.1) and 802.5 (250.6–1731.4) pg/mL, respectively; the levels in AF were significantly higher than those in MS (by paired *t* test, $P < 0.001$). Inhibin A levels in the AF and MS samples were not significantly correlated ($r = 0.19$; $P > 0.05$).

Umbilical cord serum

Cord blood was obtained from 45 pregnancies at term (37–41 weeks gestation), 24 with a female baby and 21 with a male, as evident on examination. Inhibin A was undetectable in all 45 samples, both arterial and venous. Inhibin B was undetectable in the cord serum from all female babies, but was present in serum from all 21 male babies, with no differences between arterial or venous blood levels. The median (10–90th percentile) inhibin B level in the umbilical vein was 167.4 (111.2–224.8) pg/mL. There was no association between cord inhibin B level and either gestation ($r = 0.005$; $P = 0.98$) or birth weight ($r = 0.11$; $P = 0.6$).

Discussion

Immunoreactive inhibin levels throughout pregnancy have been extensively reported using inhibin α-subunit-based assays (5–9). Serum levels of immunoreactive inhibin rise from ovulation to a peak at 9–10 weeks gestation, falling to a plateau at approximately 15 weeks and thereafter rising in the third trimester to another peak at term (5–8). A similar biphasic ontogeny for inhibin A has been recently described (24, 25). On this much larger series of MS samples than

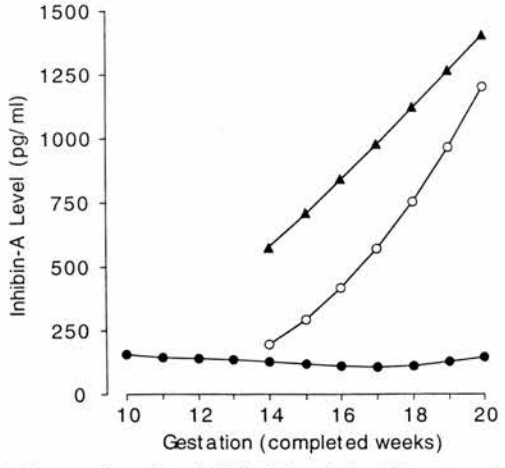

FIG. 2. Regressed median inhibin A levels in 807 maternal serum samples at 10–20 weeks gestation (●) and in 603 amniotic fluid samples at 14–20 weeks gestation (▲) and regressed median inhibin B levels in 189 amniotic fluid samples at 14–20 weeks gestation (○).

TABLE 1. Median and 10th and 90th percentile inhibin A levels (picograms per mL) in amniotic fluid from 603 chromosomally normal singleton pregnancies

	Weeks gestation						
	14	15	16	17	18	19	20
No.	73	117	87	133	137	47	9
10th percentile	158.2	183.7	206.9	212.7	322.8	282.0	489.4
50th percentile	615	680.2	728.7	997.8	1195.9	1130.7	1336.0
90th percentile	1124.6	1438.8	1389.4	2156.0	2717.4	2608.2	2084.1

TABLE 2. Median and 10th and 90th percentile inhibin B levels (picograms per mL) in amniotic fluid from 189 chromosomally normal singleton pregnancies

	Weeks gestation						
	14	15	16	17	18	19	20
No.	30	30	30	30	30	30	9
10th percentile	67.4	116.0	133.1	114.0	255.6	280.4	439.3
50th percentile	216.6	334.6	261.4	631.8	775.4	1089.2	1078.2
90th percentile	554.6	884.4	1451.8	1738.4	1887.5	2874	2482.5

TABLE 3. Median and 10th and 90th percentile inhibin A levels (picograms per mL) in maternal serum from 807 chromosomally normal singleton pregnancies

	Weeks gestation										
	10	11	12	13	14	15	16	17	18	19	20
No.	75	75	75	75	75	75	75	75	75	75	57
10th	101.4	88.8	86.8	62.3	73.8	73.2	54.2	59.5	67.3	61.2	74.1
50th	177.5	164.3	159.1	133.9	157.4	142.5	119.2	111.9	156.0	146.2	180.3
90th	290.7	277.8	253.5	212.2	295.0	261.2	189.4	200.3	291.2	277.4	327.2

previously reported, our findings confirm the biphasic profile, but show that inhibin A levels rise at 18 weeks gestation, earlier than previously reported (24). Contrary to our findings, Qu *et al.* (26) showed a steady rise in bioactive inhibin levels without the biphasic profile. It would now appear that these bioactive inhibin data have been confounded by either other FSH-regulating peptides in MS, such as activin (27), or by inadequate stripping of the very high levels of circulating sex steroids that exist in pregnancy (26). Our studies also demonstrate that inhibin B is not detectable in MS at 10–20 weeks gestation, extending the finding of a previous report that inhibin B was absent in MS up to 11 weeks gestation (25).

This is the first report of specific inhibin dimers in AF. The higher level of inhibin A in AF than in MS is consistent with existing reports of immunoreactive inhibin levels in these compartments (28, 29). It is perhaps surprising that AF levels of inhibin A are higher than those in MS because hCG, which is also secreted by the placenta, is significantly lower in AF than in serum (30, 31), consistent with secretion from the placenta into the maternal circulation. Our data suggest that either inhibin A is preferentially secreted by the placenta into the AF, rather than the maternal circulation, which is unlikely considering anatomical relationships, or that there is another significant source of inhibin during pregnancy. The lack of correlation of AF inhibin A with MS inhibin A and the differing ontogenies of inhibin A in MS and AF support the latter explanation, suggesting that different sources contribute to different compartments. Furthermore, the presence of inhibin B in AF, but not in MS, and the finding that AF levels of inhibin A and inhibin B are only weakly associated are also consistent with the presence of at least two independent sources of inhibin.

Potential sources of inhibin in pregnancy include the decidua, the fetus, and the fetal membranes, which all express inhibin subunit mRNAs (32–34). The decidua is an unlikely candidate, as it preferentially expresses β_B-subunit mRNA (32), suggesting that it would secrete more inhibin B than inhibin A, the converse of our observations. Further, if decidually derived, it might be expected that inhibins would be detectable in both MS and AF, similar to other decidual products, such as PRL and placental protein 14 (30, 35, 36), but inhibin B is not detectable in MS. It is possible that the inhibins in AF may be fetally derived, as alphafetoprotein, which is of fetal origin, is found in higher concentrations in AF than MS (37), similar to inhibin A. Previous studies of immunoreactive inhibin in cord serum concluded that the fetus was not a significant source of inhibin (5, 6, 8), and although we were unable to collect fetal blood at 10–20 weeks, we demonstrated that there is no inhibin A in the fetal circulation at term, which would argue against a fetal source for the inhibin A in AF. The fetal membranes are, therefore, the likely origin of the inhibin A in AF at the gestation periods studied. Of these, the amnion selectively expresses β_B-subunit mRNA (34), which is more indicative of activin production, but the chorion expresses mRNA for both the β_A- and α-subunits (34), consistent with inhibin A secretion.

The fetal membranes are also the probable source of the inhibin B detected in AF. We found no sex differences in AF inhibin B levels, which is not consistent with our umbilical cord data or previous midgestation data for immunoreactive inhibin (38). This would argue against a significant fetal contribution to the inhibin B content of AF, although, as for inhibin A, we were unable to collect fetal blood at the earlier gestation times, and we cannot exclude the fetus as a contributor of inhibin B to AF.

In cord serum from female babies, neither inhibin A nor inhibin B was present, but in the males, we found levels of inhibin B comparable to those in adult men (39). This differs from a previous report that no dimeric inhibins are present in cord serum (40). That Billiar and colleagues (40) found no inhibin B can be explained by the capture antibody (E4) used in their ELISA-B assay, which is an antiinhibin β_A-subunit antibody displaying no significant affinity for the inhibin β_B-subunit. The fetal testis expresses mRNA for the inhibin subunits, whereas the ovary does not (32), in keeping with inhibin B being of testicular origin, rather than from another source common to both sexes, such as the adrenals. Therefore, these data may be novel evidence that the human fetal testis secretes dimeric inhibin. It is likely that the majority of the inhibin detected in cord serum by the inhibin α-subunit assays used in previous studies (5, 6, 8) is free α-subunit (40). Although there are no sex differences in immunoreactive

inhibin levels in cord serum at term (6, 8, 41), at 26–28 weeks, immunoreactive inhibin levels are higher in males than in females (38). In light of our data, the immunoreactive inhibin findings at earlier periods of gestation may represent relative changes in inhibin B, particularly when at these times of gestation FSH levels in males are significantly lower than those in females (38, 42, 43). This sex difference in circulating FSH has been previously explained by the higher circulating levels of testosterone in males (42, 43), but it is possible that it may at least in part be due to inhibin B, being evidence of negative feedback by inhibin B on fetal pituitary FSH secretion at these periods of gestation.

In summary, our data offer some clarification of the biology of inhibin in early to midpregnancy, providing evidence to challenge the concept that the placenta is the only significant source of inhibin A in pregnancy. We suggest that the placenta secretes inhibin A primarily into the maternal circulation, whereas the fetal membranes are the likely major source of both inhibin A and inhibin B in AF. Our data also show for the first time that the fetal testis may secrete inhibin B.

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Maternal serum inhibin-A and free β-hCG concentrations in trisomy 21 pregnancies at 10 to 14 weeks of gestation

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Objective To determine the relation between maternal serum inhibin-A and free β-hCG concentrations in chromosomally normal pregnancies and to compare the two biochemical markers for their sensitivity in identifying trisomy 21 pregnancies.

Sample Inhibin-A and free β-hCG were measured in maternal serum samples from 800 chromosomally normal singleton pregnancies at 10 to 14 weeks of gestation and 76 singleton pregnancies with fetal trisomy 21.

Results In the normal group maternal serum inhibin-A was significantly associated with both maternal weight and gestational age ($F = 11{\cdot}2$, $P < 0{\cdot}0001$). In pregnancies with trisomy 21 the maternal serum inhibin-A and free β-hCG concentrations were significantly increased (mean difference inhibin = 0·51 SD, $F = 18$, $P < 0{\cdot}0001$ and mean difference free β-hCG = 1·13 SD, $F = 80$, $P < 0{\cdot}0001$). For a 5% false positive rate, the sensitivity of maternal serum free β-hCG in identifying pregnancies with trisomy 21 was 28·9% compared with 12·8% for maternal serum inhibin-A. Delta inhibin-A was significantly associated with delta-free β-hCG ($r = 0{\cdot}345$, $P < 0{\cdot}01$) and the deviation from the normal mean for free β-hCG was significantly greater than the deviation for inhibin-A ($t = 4{\cdot}0$, $P < 0{\cdot}0001$). For a 5% false positive rate, the sensitivity achieved by combining information from delta inhibin-A and delta free β-hCG was similar to the sensitivity of free β-hCG alone (30·3% compared with 28·9%)

Conclusion At 10 to 14 weeks of gestation fetal trisomy 21 is associated with increased maternal serum inhibin-A and free β-hCG levels. However, the degree of elevation of inhibin-A is less than that of free β-hCG, and there is a significant association between levels of the two proteins. The sensitivity for trisomy 21 achieved with the combination of maternal serum inhibin-A and free β-hCG is not significantly different from that achieved with maternal serum free β-hCG alone.

INTRODUCTION

Inhibin is a heterodimeric glycoprotein formed by the combination of an β-subunit with either a β_A-subunit (inhibin-A) or a β_B-subunit (inhibin-B); additionally there are circulating nonbioactive free-subunits[1,2]. In pregnancy the major source of inhibin in maternal serum is thought to be the placenta[3].

The first immunoassay for inhibin used antibodies directed at the β-subunit only and could therefore not distinguish between inhibin A, inhibin B and the free-β-subunits[1]. More recently, immunosorbent assays (ELISA) specific for each dimer have been introduced[4,5]. Studies using these specific assays have reported that inhibin-B is not detectable in maternal serum in early pregnancy[6], whereas the levels of inhibin-A which mirror those found with the nonspecific assays, increase with gestation to reach a peak at 10 to 12 weeks; they subsequently decline to a plateau in the second trimester but they rise again in the third trimester to maximum levels at term[6–8].

Studies using the nonspecific inhibin (immunoreactive) assays have reported that in trisomy 21 maternal levels are increased in the second[9,10] but not in the first trimester of pregnancy[11]. Studies with the specific assay have reported that levels of inhibin-A in trisomy 21 are increased both during the first and the second trimesters[12,13]. Furthermore, in the second trimester inhibin-A is more sensitive than immunoreactive inhibin in the detection of trisomy 21[14], and in the second trimester there is no significant correlation between inhibin-A and free β-hCG so that when data from both proteins were combined inhibin-A

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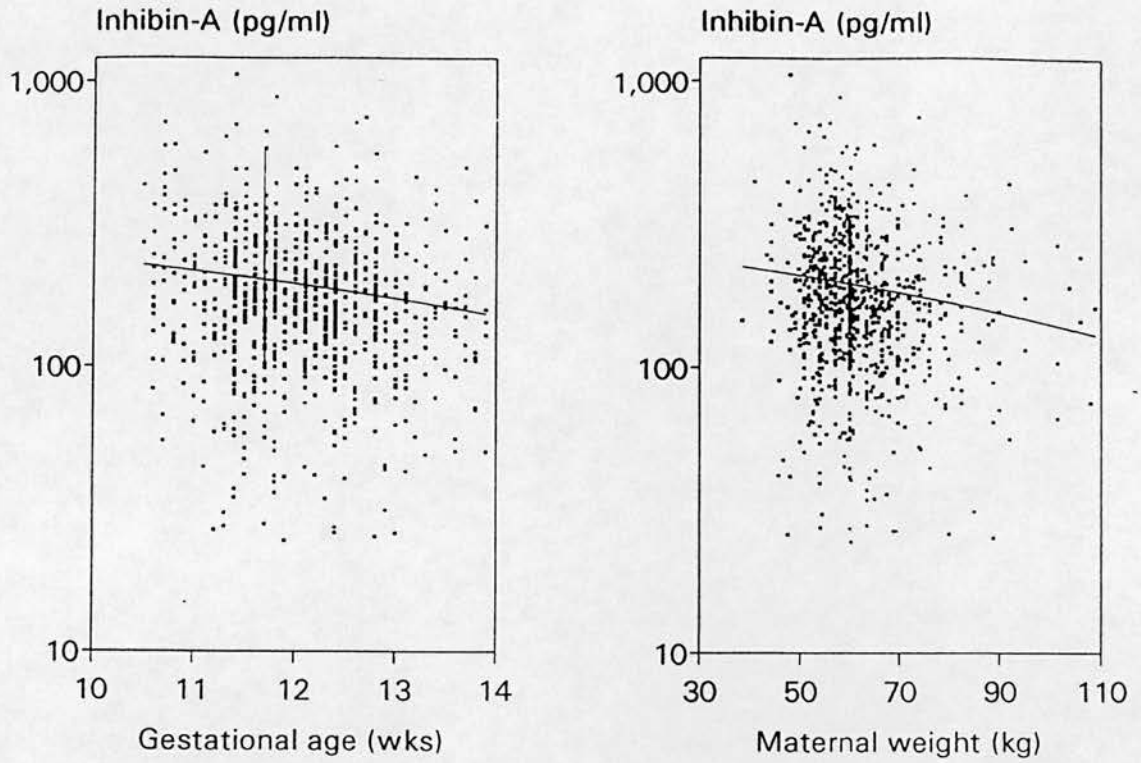

Fig. 1. Maternal serum inhibin-A concentration in 800 normal controls in relation to maternal age and maternal weight.

provided a 20% increase in the detection of trisomy 21 for a given fixed false positive rate[15].

The aim of this study was to examine the level of maternal serum inhibin-A in first trimester trisomy 21 pregnancies and to explore whether this placental product may also improve the sensitivity of free β-hCG in the detection of trisomy 21 at this gestation.

METHODS

Serum free β-hCG and inhibin-A concentrations were measured in blood samples obtained immediately before ultrasound examination in women with singleton pregnancies attending the Harris Birthright Research Centre for Fetal Medicine. The patients were self-referred for screening for fetal trisomy 21 by maternal age and fetal nuchal translucency thickness at 10 to 14 weeks of gestation. The assays were carried out in samples from 76 pregnancies with trisomy 21 and 800 chromosomally normal controls, over the same range of maternal age and gestation. Written consent was obtained from all the patients and the study was approved by the hospital ethics committee.

Maternal serum inhibin-A was determined retrospectively after samples had been stored at –20 °C for up to 12 months. Maternal serum free β-hCG was determined prospectively after samples had been stored overnight at 4°C, or when samples were taken on Friday, at –20°C for two days. Biochemical analysis for both analytes was performed without knowledge of fetal karyotype.

Inhibin-A was measured using a specific two-site enzyme linked immunosorbent assay (ELISA) as previously described[16] but with some minor modifications. Briefly, serum samples and standards were mixed with 1% (final w/v) hydrogen peroxide for 30 minutes prior to the assay. The assay used an immobilised anti inhibin-A subunit monoclonal antibody covalently bound to hydrazide microplates (Avidplate-HZ) as the capture antibody. The second antibody is the Fab fraction of a mouse anti β-inhibin subunit monoclonal antibody conjugated to alkaline phosphatase. Detection was afforded by colour change after adding alkaline phosphatase substrate (P-NPP KPL, Maryland, USA). Recombinant 32 kD human inhibin-A (Genentech) was used for standards with the results expressed as pg/mL. The sensitivity of the assay was 39 pg/mL and the intra and inter-plate coefficients of variation were 4·3% and 5·6%, respectively.

The concentration of maternal serum free β-hCG was determined using an immunoradiometric assay (CIS, Paris, France). The sensitivity was 0·15 ng/mL and intra- and inter-assay coefficients of variation were 3·1% and 5·7%, respectively.

Table 1. Median maternal weight, maternal serum inhibin-A and free β-hCG concentration (median and 90% CI) in normal controls in relation to gestation. CI = confidence intervals

Gestation (weeks)	*n*	Maternal weight (kg)	Inhibin-A Median	Inhibin-A 90% CI	β-hCG Median	β-hCG 90% CI
10	52	61	204	67–481	44	16–120
11	318	62	173	50–373	41	15–119
12	325	62	168	62–382	34	13–98
13	105	62	146	50–313	30	12–91

Table 2. Median maternal weight, maternal serum inhibin-A and free β-hCG concentration (median and 90% CI) in 76 pregnancies with trisomy 21 in relation to gestation.

Gestation (weeks)	*n*	Maternal weight (kg)	Inhibin-A Median	Inhibin-A 90% CI	β-hCG Median	β-hCG 90% CI
10	3	62	354	244–463	91	81–121
11	27	65	274	102–483	66	32–192
12	31	63	213	37–571	60	21–210
13	15	64	217	85–336	66	20–152

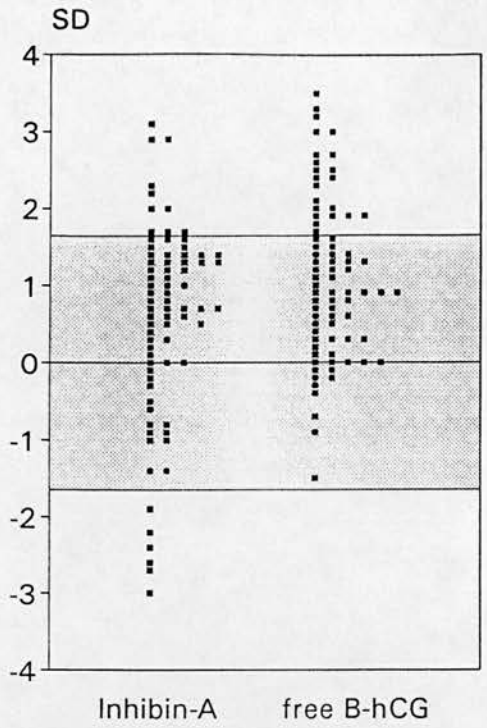

Fig. 2. Maternal serum inhibin-A and free β-hCG concentration in 76 pregnancies with trisomy 21 expressed as the number of standard deviations by which values differed from the appropriate normal mean for gestation and maternal weight.

The distribution of maternal serum inhibin-A measurements was examined and found to be skewed. To normalise the distribution, log transformation was applied. Regression analysis was used to determine the inter-relation of log transformed maternal serum inhibin-A measurements with fetal crown–rump length and maternal weight. The equation which described the relation was then used to calculate for each pregnancy the number of standard deviations by which the concentration differed from the appropriate normal mean for maternal weight and gestation (delta value). Delta values for maternal serum free β-hCG were calculated using the findings from a previous study[17]. Regression analysis was applied to examine the relation between delta inhibin-A and delta free β-hCG and paired t-test was used to compare deviations.

RESULTS

The median maternal age was 34 years (range 15–47) and the median gestation by crown-rump length was 12 weeks (range 10–14). In the control group maternal serum $\log_{10}$ inhibin-A was significantly associated with both maternal weight and gestational age (Fig. 1, $\log_{10}$(inhibin-A) = 2·897 – 0·0423 × gestation [weeks] – 0·002786 × maternal weight [kg], SD 0·250, SE for gestation coefficient = 0·0119, SE for maternal weight coefficient = 0·000876, R = 0·166, F = 11·2, P < 0·0001). To allow comparison with previous and future studies, basic information on actual maternal serum inhibin-A measurements for normal and affected pregnancies is summarised in Tables 1 and 2.

In pregnancies with trisomy 21 the maternal serum inhibin-A and free β-hCG concentrations were significantly increased (Fig. 2, mean difference inhibin-A = 0·51 SD, F = 18, P < 0·0001 and mean difference free β-hCG = 1·13 SD, F = 80, P < 0·0001) and the increase was constant throughout the gestational range of the study population (Table 2, r = 0·003, P = 0·98 and r = 0·132, P = 0·26, respectively). Delta inhibin-A was significantly associated with delta free β-hCG (Fig. 3, r = 0·386, P < 0·0001 and Fig. 4, r = 0·308, P < 0·01), but the deviation from the normal mean for free β-hCG was significantly higher than the deviation for inhibin-A (t = 4·0, P < 0·0001). Delta inhibin-A was above the 95th centile in 10 (12·8%) cases, while delta free β-hCG was above the 95th centile in 22 (28·9%) cases. For the same false positive rate (5%), the combined information from delta inhibin-A and delta free β-hCG identified 23 (30·3%) of the pregnancies with trisomy 21 which is not significantly different from the detection of trisomy 21 by free β-hCG alone (28·9%, χ^2 = 0·023).

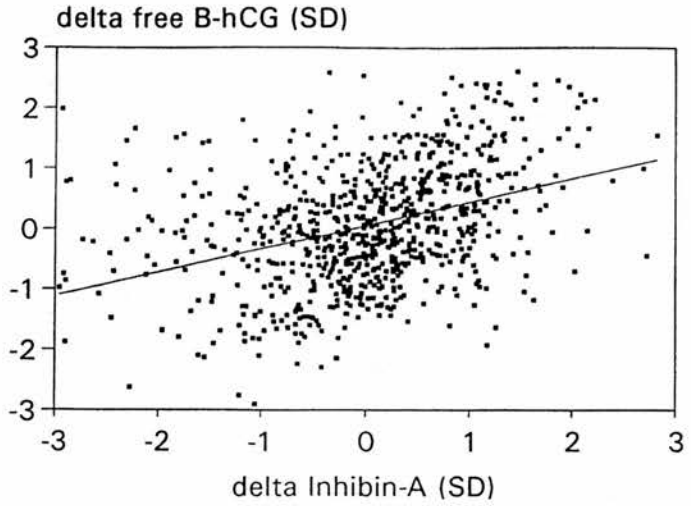

Fig. 3. Maternal serum inhibin-A concentration in 800 normal controls in relation to maternal serum free β-hCG concentration. Individual values for both biochemical markers were expressed as the number of standard deviations from the appropriate normal mean for gestation and maternal weight

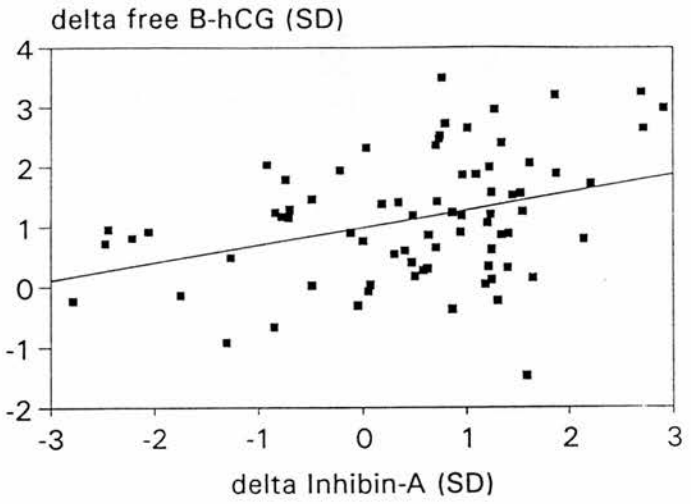

Fig. 4. Maternal serum inhibin-A concentration in 76 pregnancies with trisomy 21 in relation to maternal serum free β-hCG concentration. Individual values for both biochemical markers were expressed as the number of standard deviations from the appropriate normal mean for gestation and maternal weight.

DISCUSSION

The findings of this study demonstrate that in the first trimester of trisomy 21 pregnancies maternal serum inhibin-A is increased; for a 5% false positive rate, 12·8% of pregnancies with trisomy 21 can be identified. However, there is a significant association between inhibin-A and free β-hCG, and the latter is more sensitive in detecting trisomy 21 (28·9% for a 5% false positive rate). Studies in the second trimester have demonstrated that addition of inhibin-A to maternal serum screening with alpha-fetoprotein and free β-hCG provides a 20% increase in the sensitivity of the test[15]. Our findings indicate that this is not the case in the first trimester of pregnancy; the addition of inhibin-A to screening with free β-hCG did not significantly increase the sensitivity.

The gestational age-related differences in the relation between free β-hCG and inhibin-A[15] in maternal blood suggest that with advancing gestation there is a change in the regulation of these glycoproteins. Trophoblast, in particular syncytiotrophoblast, is thought to be the primary source of both inhibin-A and hCG[18,19]. It is also apparent from *in vitro* studies that the secretion of hCG and inhibin may be interdependent[20,21]. hCG stimulates inhibin secretion from the trophoblast, and inhibin suppresses GnRH-stimulated hCG secretion from term but not first trimester, trophoblast cell cultures. While inhibin levels fall from a peak at 10 to 12 weeks to a plateau at 15

weeks[8,22] the fall is not as profound as that for hCG[22]. hCG levels remain low in later pregnancy when inhibin levels rise to a maximum levels at term[7]. Therefore, it would appear that the regulation of inhibin is hCG-dependent in early pregnancy but hCG-independent from the second trimester onwards.

The sensitivity of screening for trisomy 21 by maternal age and fetal nuchal translucency thickness at 10 to 14 weeks of gestation is about 80% for a 5% false positive rate[23]. Addition of maternal serum free β-hCG increases the sensitivity to 85%[17]. Although we have not specifically examined the combination of inhibin-A and nuchal translucency thickness (with or without free β-hCG), the data presented here suggest that inhibin-A would not expect to improve the sensitivity of first trimester screening by maternal age, fetal nuchal translucency thickness and maternal serum free β-hCG and so would not be a valuable marker at this stage of pregnancy.

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